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A BIOLOGICAL WASTE WATER MONITOR STUDY USING DIFFERENTIAL LASER--ETC(U)
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UNCLASSIFIED SSI-2015-F AFOSR-TR-79-1278 NL

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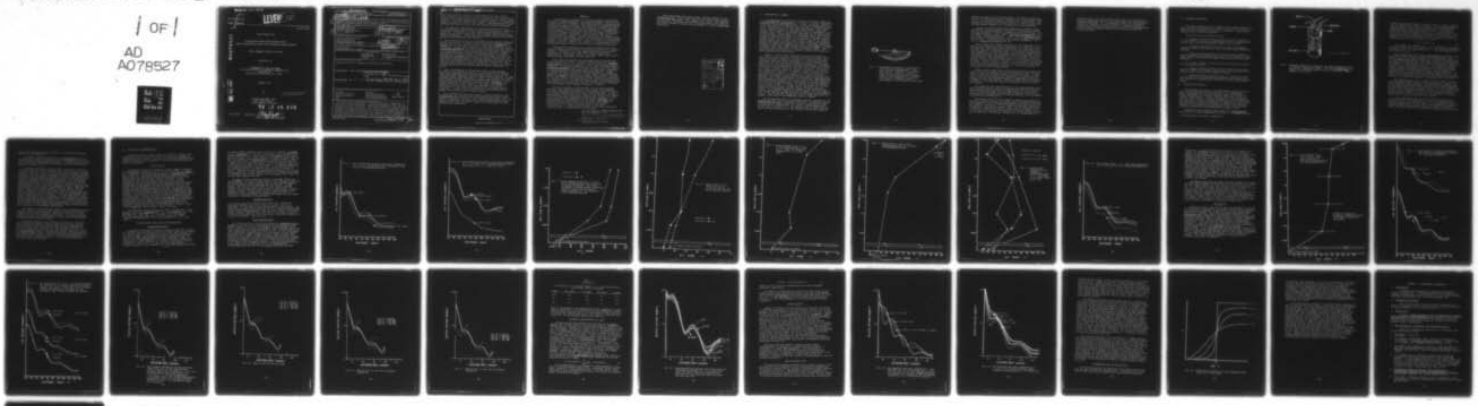

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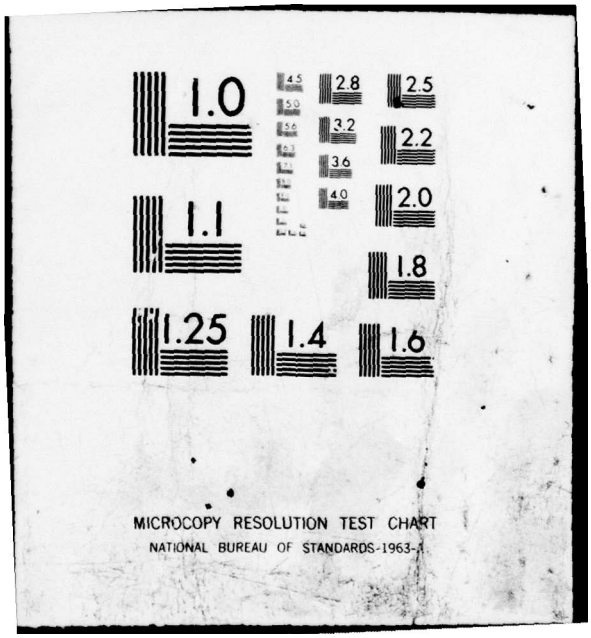
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SCIENCE SPECTRUM

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Final Report For

A BIOLOGICAL WASTE WATER MONITOR STUDY USING DIFFERENTIAL LASER LIGHT SCATTERING FROM BACTERIA

AFOSR CONTRACT F49620-78-C-0096

Submitted to

DEPARTMENT OF THE AIR FORCE
AIR FORCE OFFICE OF SCIENTIFIC RESEARCH (NL)
Building 410
Bolling Air Force Base, DC 20332

October 1979

By

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SSI-2015-F Approved: Philip J. Wyatt

Philip J. Wyatt, President

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

A ninety-minute bioassay of selected toxicants (monomethylhydrazine, dimethylhydrazine, nitrosodimethylamine, JP4, and JP8) in water has been confirmed. The method consists of measuring the differential light scattering (DLS) patterns of exponential phase bacterial suspensions in water containing the selected toxicant relative to a toxicant-free control suspension. The suspensions are illuminated by a fine laser beam and the light scattered as a function of angle with respect to the incident beam is recorded—

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Block 20. Abstract (cont.)

(the DLS pattern). Relative to the bacteria in the control, toxicant-free, suspension, bacteria affected by the toxicants may change their shape, average size, size distribution, and/or number density. Any such changes will in general result in a changed DLS pattern relative to that of the control. The measurement of such differences forms the basis for the present bioassay technique.

The bacterial suspensions are usually prepared from a bacterial chemostat source driven by paristaltic pumps of a Technicon manifold. Throughout an 8 hour sampling period cultures will generally not vary by more than 20% in number density and negligibly in morphology. Within the ninety minute assay period, number density variations are less than 5%.

Each selected toxicant was challenged against several different bacterial strains. A highly sensitive strain of Staphylococcus aureus (SSI 41) was used for the jet fuels and monomethylhydrazine. It was also responsive to the other toxicants chosen. Klebsiella pneumoniae (Kp 101) was most sensitive for the assay of nitrosodimethylamine and E. coli ATCC 13473 was highly sensitive to dimethylhydrazine as well as monomethylhydrazine. Studies performed with mixed cultures of diverse species (for example E. coli and S. aureus) showed that at equal number density, the DLS pattern was effectively destroyed thereby reducing the visibility of toxicant effects. The DLS response of a mixed culture was only the response of the most sensitive constituent, thus rendering the remaining species superfluous, at best, and sensitivity reducing (via decreasing the DLS pattern features), at worst.

With the exception of JP4 and JP8, both of which produce monotonic dose-response effects for both growth and morphology, the other compounds often affected the bacterial cells in two distinct modes, depending upon the assay strain selected: at high concentrations (less than about 5 μg/ml), the effects were inhibitory and/or produced significant morphological changes. At low concentrations, the compounds acted as growth stimulants - at least during the ninety minutes of the assay. For different bacterial strains, this effect was reversed, with growth stimulation at the higher and inhibition at the lower concentrations. A ninety minute response was usually detectable at about 5 parts per billion of the toxicant.

Most measurements were made using a scanning version of the Differential III, though those involving JP4 and JP8 were performed with the fixed array, fully computerized system. The completely automated, continuous flow DLS 800 system could not be used, as USDA staff refused to permit any carcinogens in their microbiology laboratory.

Technical Information Officer

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I. INTRODUCTION & SUMMARY

The detection of all and any toxicants in water, unlike the identification and quantitation of a specific toxicant, represents a problem of enormous proportions. So many substances, combinations, and metabolites thereof exist that may be potentially toxic to the exposed party, that their mere enumeration is even impossible. Indeed, because of cumulative dose effects, many substances once thought to be harmless must now be considered in the toxicant class. Since there is no economical way by which means an essentially infinite number of biochemical tests could be performed to detect the presence of all potential toxicants, the EPA (among others) have in recent years proposed the use of various living systems such as fish, brine shrimp, plankta, and aufwuchs. Though such higher life systems will show a reasonably sensitive response to very broad ranges of toxicants, their time of response is usually very slow. Times between exposure and effect can often be several days, especially at very low toxicant levels. For large flowing water supplies or treatment plants such delays are both impractical and intolerable.

Ideally, a broad screen bioassay should yield results in real-time or close enough to the time of sampling to make retention of the water a practical possibility. Such rapid bioassay techniques have been under development at Science Spectrum for some years and have now been successfully applied in the present study to a set of five toxicants (monomethylhydrazine, nitrosodimethylamine, dimethylhydrazine, JP4 and JP8) of particular interest to the Air Force Office of Scientific Research. The method uses highly sensitive bacterial strains produced continuously under chemostatic conditions. Aliquots of these bacteria are combined with water samples, incubated for 90 minutes, and then examined for changes in their differential light scattering (DLS) patterns. The latter measurement is performed by illuminating the post-incubation suspension with a fine laser beam, then measuring and recording the intensity of light scattered from the sample as a function of scattering angle. A schematic of the DLS measurement is shown in Fig. 1. Changes of the DLS pattern of toxicant-exposed bacteria relative to the control suspension will be created by corresponding physical changes in the bacteria themselves. Such changes, induced by the toxicants, may be changes in cell number density, average cell size, structure, or size distribution. The DLS pattern changes may then be quantitated and displayed in the form of dose-response curves or tables.

The measurements were all performed using two versions of the Differential III light scattering photometer. An earlier version produces DLS patterns by means of a single photomultiplier that is mechanically scanned about the subject cuvette between about 30° and 130° in steps of 1°. The scanning time is about five seconds during which period the 100 intensity values are digitally

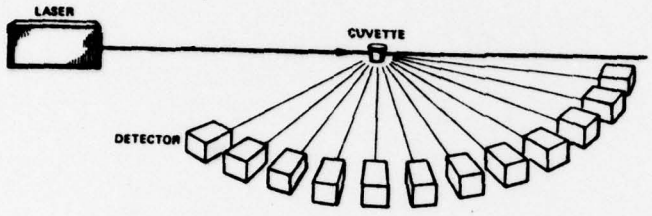


Fig. 1 - A schematic diagram of a differential light scattering measurement. The laser source illuminates a suspension of cells in a cuvette. Lying in a plane are the array detector elements, each of which measures the scattered intensity at a different angle, θ, with respect to the incident laser beam.

encoded and stored in the system memory. The newer version consists of a fixed array whose 15 elements are equidistantly spaced in sin $\theta/2$ and span the range from about 30° to about 130°. The elements are multiplexed 32 times during 15 second intervals permitting digital suppression of spurious noise contributions.

Several bacterial strains were examined for maximum linear sensitivity to the compounds of interest. Thresholds for detection and the optimal bacterial strains used were as follows: Nitrosodimethylamine, < .006 µg/ml, Klebsiella pneumoniae Kp 101 (3 hr. assay); monomethylhydrazine, < .004 µg/ml, Escherichia coli ATCC 13473; dimethylhydrazine, < .004 µg/ml, E. coli ATCC 13473; JP4 and JP8, < .05 water soluble fraction/ml (estimated < .001 µg/ml), Staphylococcus aureus SSI 42.

Many strains were broadly sensitive to the compounds of interest, but generally exhibited some degree of anomalous, non linear response. The water soluble fraction of the jet fuels, JP4 and JP8, produced a monotonic effect on the DLS growth and morphology parameters. On the other hand, the hydrazine compounds and nitrosodimethylamine would generally produce high growth stimulation effects at high concentrations of the compounds, but at low concentrations, these compounds would tend to act as inhibitors during a typical 90 minute assay period. At extremely low concentrations (< .0001 µg/ml) of course, the DLS patterns produced are the same as the plain water controls. For different assay organisms, the growth inhibition occurred at high concentrations and stimulation at small concentrations.

Mixed cultures were found to be unsatisfactory for assay purposes since their combined DLS pattern generally lacked the features of those of the pure species, contributing cells. If only one of a two species mixture was sensitive to a particular compound, then its DLS response was greatly diminished by the mere presence of the other species. Such a response would be completely absent if the sensitive species represented less than 10% of the mixture.

The stability and reproducibility of the chemostatically produced bacteria were so great on a day-to-day basis, that initial concentration densities could be predicted at any time within 20%. An analytical means by which the changes in bacterial concentration at subsequent times following exposure to the compounds of interest was established.

The ease by which the DLS method can detect the presence of potential toxicants makes it an almost perfect bioassay technique. There appears to be no comparative bioassay method that is as simple, rapid, or practical. The technique has now been completely automated via the DLS 800 system. This latter device may be used "on-stream", sampling the source waters every 90 seconds, or it may be used to assay discrete samples from various diverse sources at the same rate. Before such a system can be implemented in a

practical sense, one final task remains, viz. the correlation of the DLS methodology with the more conventional and time-consuming bioassay techniques. It is recommended that this correlation project be initiated as soon as possible. Probable savings to both the Air Force and industry by the adoption and implementation of DLS bioassay techniques could exceed tens of millions of dollars per year.

II. RESEARCH OBJECTIVES

The basic objective of the program was to collect data that would confirm the DLS method as a rapid, sensitive, and simple bioassay for five compounds of potential occurrence in waste water supplies near Air Force facilities.

The specific work statement follows:

a. Screen selected bacterial assay strains for sensitivity to the following toxicants: dimethylhydrazine (DM), monomethylhydrazine (MH), nitrosodimethylamine (NDMA), JP4, and JP8.

b. Generate dose-response curves for both morphological change and growth inhibition for each strain/toxicant combination selected.

c. Interpret quantitatively the recorded dose-response effects exhibited by the changed DLS patterns, the changes in growth rates, induced morphological changes and variations in size distribution.

d. Develop a means to measure absolute bacterial concentrations using DLS methods.

e. Examine toxicant effects on mixed cultures of selected bacterial strains and compare results with those produced individually on pure cultures of the combined strains.

f. Establish minimal response times for various toxicants upon selected bacterial strains and determine the practicality of such results for establishing a rapid and accurate screening procedure.

III. METHODS AND MATERIALS

The bioassay was based on the differential light scattering (DLS) method described in the references.[1-4]* Exponential phase suspensions of selected bacterial strains are exposed to waters containing the toxicants (DM, MH, NDMA, JP4 or JP8) at various concentrations. Control specimens are also prepared at the same time. After a 90 minute incubation, cuvettes containing the toxicant and control suspensions are examined in a fine laser beam and their DLS patterns recorded. Changes in the patterns relative to the controls are quantitated on the basis of growth inhibition/stimulation and morphological changes, if any.

Exponential phase bacteria were produced in Bactisources™, one of which is shown schematically in Fig. 2. An aliquot from an

* References may be found in Appendix E.

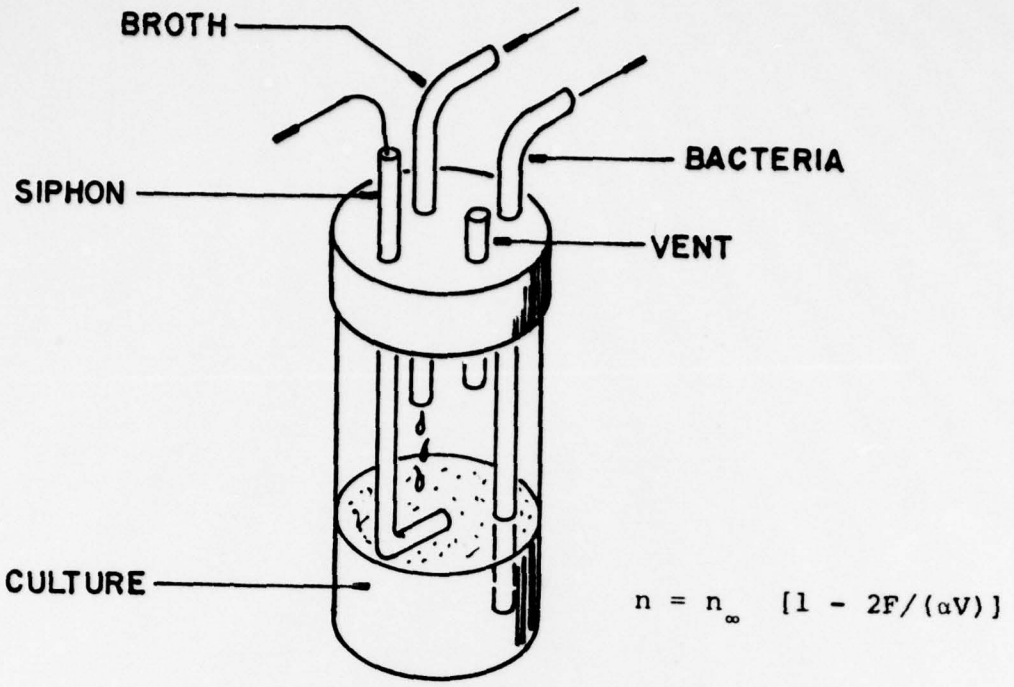


Fig. 2 Schematic layout of a chemostat to produce bacterial cells at constant exponential phase. The unit, Bactisource", is placed in a constant temperature water bath with a small magnetic bar stirrer.

overnight culture was added to sterile broth in a water jacketed (38° C) magnetically stirred container. The relative amounts of overnight culture and broth were determined from chemostatic considerations so that final concentrations of the order of 10^7/ml were continuously generated. If F is the flow rate at which fresh broth is added to the fixed chemostat volume, V, and the rate at which the bacterial suspension is removed from the chemostat (in order to maintain the fixed volume), then the cell density present in the chemostat is n, where

$$n = n_\infty [1-2F/(\alpha V)] \qquad (1)$$

The maximum cell density is n_∞ and corresponds to the density limit in the static situation and α is the reciprocal bacterial generation time. As long as $2F/(\alpha V) < 1$, the cell density will be constant in time.

Measurements were made using a Differential III system incorporating a photomultiplier scanner[5] or a fixed array.[4] The former systems were used predominantly, but have no inherent signal processing capabilities. Any dust or debris contributions to the signal occurring during a scan appear in the form of distorted DLS patterns. The array system, on the other hand, does remove digitally the contributions of debris. A completely automated system (DLS 800) including both chemostats, fixed array, sample mixing hardware (Technicon AutoAnalyzer), and incubator was planned for use in this project for demonstration purposes. The Department of Agriculture, who had earlier agreed to use of this system for this project, later reversed their decision and refused access to the system if potential carcinogens were to be used.

The methylhydrazine (MH), dimethylhydrazine (DM), and nitrosodimethylamine (NDMA) were purchased from Aldrich Chemical Company as reagent grade chemicals. (Discussions and comments made at the AFOSR Life Sciences Directorate Review of Basic Research in Environmental Protection and Toxic Hazards, held in Los Angeles in January, 1979 indicated considerable doubt as to the actual purity of the first two reagents.) The two jet fuels were sent from Wright-Patterson AFB in screw cap, one pint metallic containers. Their specific origin and purity were unknown.

Unless otherwise noted, all stock solutions were made up fresh on the day of the experiment. Units of concentration are expressed as parts per million (ppm), or its equivalent, µg/ml on a weight/weight basis. On the day of the experiment, the proper volume of chemical was measured by pipette into a volumetric flask, the flask filled to the mark with deionized 0.2 µm-filtered

water, and the stock solution placed in a dark screw cap glass bottle in a refrigerator.

Bacterial cultures generated by the Bactisources™ at 0.16 ml/min were collected for several minutes and added to 15.2 ml of chemical-dosed deionized/filtered water. Depending upon the type of bacteria used, the standard bacterial inoculum was fixed between 0.3 ml and 1.0 ml.

The three carcinogens (MH, DH, NDMA) were added to the deionized/filtered water by combining 15 ml water with 0.2 ml chemical stock solution. The jet fuels presented some unique problems. Initially fuels were added to waters and shaken vigorously before decanting. This procedure invariably resulted in the formation of micelles whose presence and relatively uniform size distribution tended to overwhelm the bacterial suspension. The DLS patterns recorded, therefore, were often more characteristic of the micelle/bacteria mixture than the bacteria as affected by the fuels. Jenkins and his group at Berkley emphasize that the important jet fuel parameter is its water soluble fraction (WSF). They have constructed a reactor that is used to extract the WSF of the fuels. Samples of such reacted waters were finally obtained from their laboratory, but were found to be heavily contaminated with unknown bacteria (about 5×10^6/ml). At these high levels, bacterial metabolites would be expected to cause significant effects, perhaps even greater than the WSF itself. Accordingly, the WSF's were extracted by placing about 20 ml of the fuels in sealed cellophane dialysis bags within 500 ml containers of distilled water for 24 hours. The WSF easily permeated the bags and provided the stock solutions for subsequent experiments.

The amounts of the toxicants present were calculated starting with the figures supplied by the reagent manufacturer (Aldrich Chemical Co.) and the subsequent dilutions. The jet fuels were assumed to be at full strength as received. No attempt was made to determine the actual WSF present in these fuels, the Berkeley estimates proving utterly useless.

Bacterial enumeration was achieved by means of a Petroff-Hauser counting chamber. Such an enumeration yielded absolute cell numbers per milliliter rather than the more commonly used colony forming units (CFU) per ml. DLS methods are excellent means for enumerating relative bacterial number densities. Since the Bactisources™ could be started each day at essentially identical number density levels, a single Petroff-Hauser measurement for each species used could be used for subsequent determinations of bacterial number densities based on the DLS methodologies described in the next section.

IV. RESULTS AND RECOMMENDATIONS

The basic results of this important study have already been summarized in Sec. I of this report. This section presents an elaboration of those data. Only selected data are presented here, generally typical of the hundreds of results obtained during the contract's progress.

ASSAY STRAINS

In addition to the most versatile (widest range of response to contaminants) bacteria in use by Science Spectrum, S. aureus 41, a sampling of gram negative E. coli and K. pneumoniae bacteria was carried out. The gram negative bacteria generally have a higher ratio of lipopolysaccharide/peptidoglycan as cell wall constituents than do the gram positive bacteria. It was anticipated that this cell wall composition would be more susceptible to (a) the detergent and chaotropic action of highly charged substituted amines and nitroso-compounds, and (b) more susceptible to penetration by these molecules to the sites of nucleic acid and protein synthesis such that the bacteria could mutate or suffer immediate cytotoxic effects, expressed (per DLS parameters) as size, shape, number density, and refractive index changes. In particular, from previous work[6], E. coli ATCC 13473 was known to be an excellent assay organism for the nitrosourea carcinogen BCNU. Another organism K. pneumoniae 101 produced via plasmid insertion for Science Spectrum by the Bethesda Research Laboratories from the parent type K. pneumoniae 886 has a strikingly different colonical morphology than K 886 in that discrete colonies without "mucoid" spreading are evident. This "bald" Klebsiella seemed another likely candidate.

The organisms assayed against MH, DMH, and NDMA were (i) S. aureus 41, (ii) K. pneumoniae 886, (iii) K. pneumoniae 101, (iv) E. coli 3643 (Thy^-, Thi^-), (v) E. coli K-12, (vi) E. coli B-8, (vii) E. coli 2480, and (viii) E. coli ATCC 13473. Organisms assayed against JP4 and JP8 included (i), (ii), and (viii).

DOSE-RESPONSE CURVES AND INTERPRETATIONS

Monomethylhydrazine

E. coli K-12 yielded a "linear" response to MH from 12 µg/ml to 1 µg/ml. The term "linear" is used in this report as shorthand for the log (dose) versus response curve which will exhibit, in its linear region, an increasing monotonic response or "score" from low to high concentration, for some reasonable range. It should also be emphasized that the score referred to is based on the growth, G, and morphology, M, dichotomy discussed in the references.[3,4] When G or M, or the sum of the two yields a linear response, it is so labeled. E. coli 2480 similarly yielded

an almost linear response from 12 µg/ml to 1.0 µg/ml. S. aureus 41, K. pneumoniae 101 (Kp 101) and E. coli 13473 displayed much greater sensitivity, to 0.004 µg/ml, but S. aureus 41 was linear only from 0.004 to 0.1 µg/ml, whereas at higher concentrations of MH, the compound caused stimulation of bacterial growth. This is illustrated in Fig. 3 wherein the DLS patterns for the control, 1 µg/ml, and 0.01 µg/ml are shown. Kp 101 was almost linear from 0.004 µg/ml to 12 µg/ml and serves well as a screening organism for monomethylhydrazine. E. coli 13473 is as sensitive as Kp 101 and perhaps superior in terms of linear response as shown in Fig. 4. Figures 5-8 show the dose-response plots of S 41, Kp 101 and E. coli 13473 to MH, emphasizing the low concentration range of interest (ppb), except for Kp 101 and E. coli 13473, where the linear range response is wider.

E. coli B-8 and Kp 886 were tested a few weeks after the above strains, but their control growth curves were not as good in the essentially pure water medium used in the standard protocol. Further testing of these organisms was not pursued. Kp 886, however, may deserve further study if some type of nutrient addition to the water would prove useful in the future. For the present, however, sensitivities are more than sufficient using unmodified water. E. coli B-8, though sensitive to MH, is difficult to maintain in the laboratory and should not be further considered a practical bioassay strain.

Dimethylhydrazine

S 41, and E. coli K-12 exhibit poor sensitivity to DMH with measurable response only at 10 µg/ml or higher. They are, therefore, not suitable as assay organisms for this toxicant. E. coli 2480, E. coli 13473 and Kp 101 all show excellent sensitivity to DMH, down to approximately 0.004 µg/ml, but with a very limited linear response range between 0.001 and 0.01 as shown in Fig. 9. At "high" concentrations, in the ppm range, the growth stimulation effects are present. (See Fig. 10).

Nitrosodimethylamine

An initial survey of the five E. coli, two Klebsiella and S. aureus 41 vs. NDMA, with the standard 90 minute protocol showed erratic results. For S 41, for example, a concentration of 18 µg/ml showed only a slight "morphology" effect, whereas higher and lower concentrations showed no effect at all. Kp 101 and E. coli 3643 showed some responsiveness, but the overall erratic results suggested that a longer incubation period might be called for, especially since NDMA, a known carcinogen, may be acting through mutation and require more than a few generation times for complete phenotypic expression of the mutation. With purines and amino acids added to the incubation medium, as had been done previously for E. coli 13473 vs. BCNU and S 41 vs. sulfonamides,[6,7] results indicated that Kp 101 and E. coli 2480 were the most

Fig. 3 - The recorded DLS patterns from water suspensions of *S. aureus* 41 incubated for 90 minutes at two levels of monomethylhydrazine.

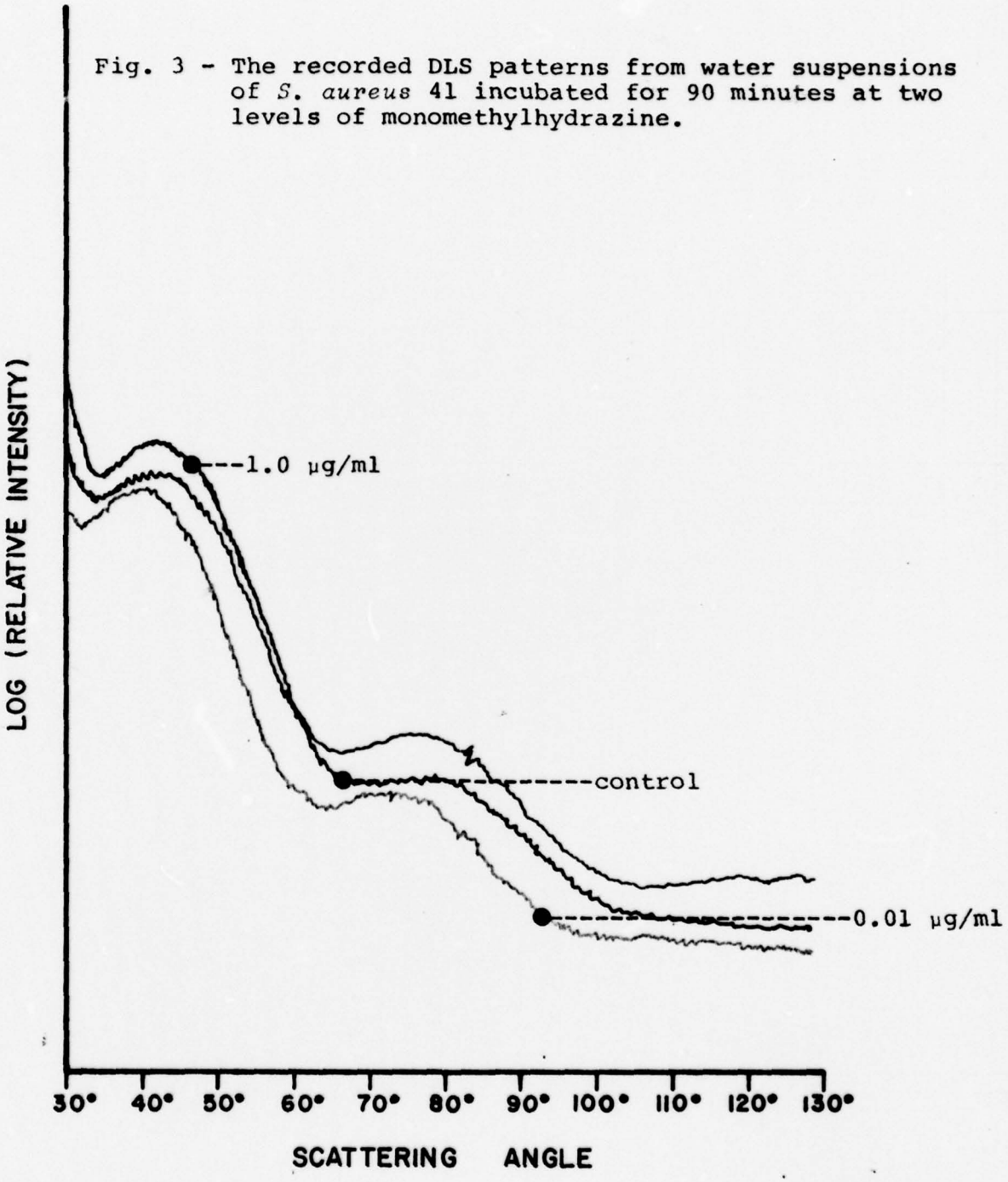
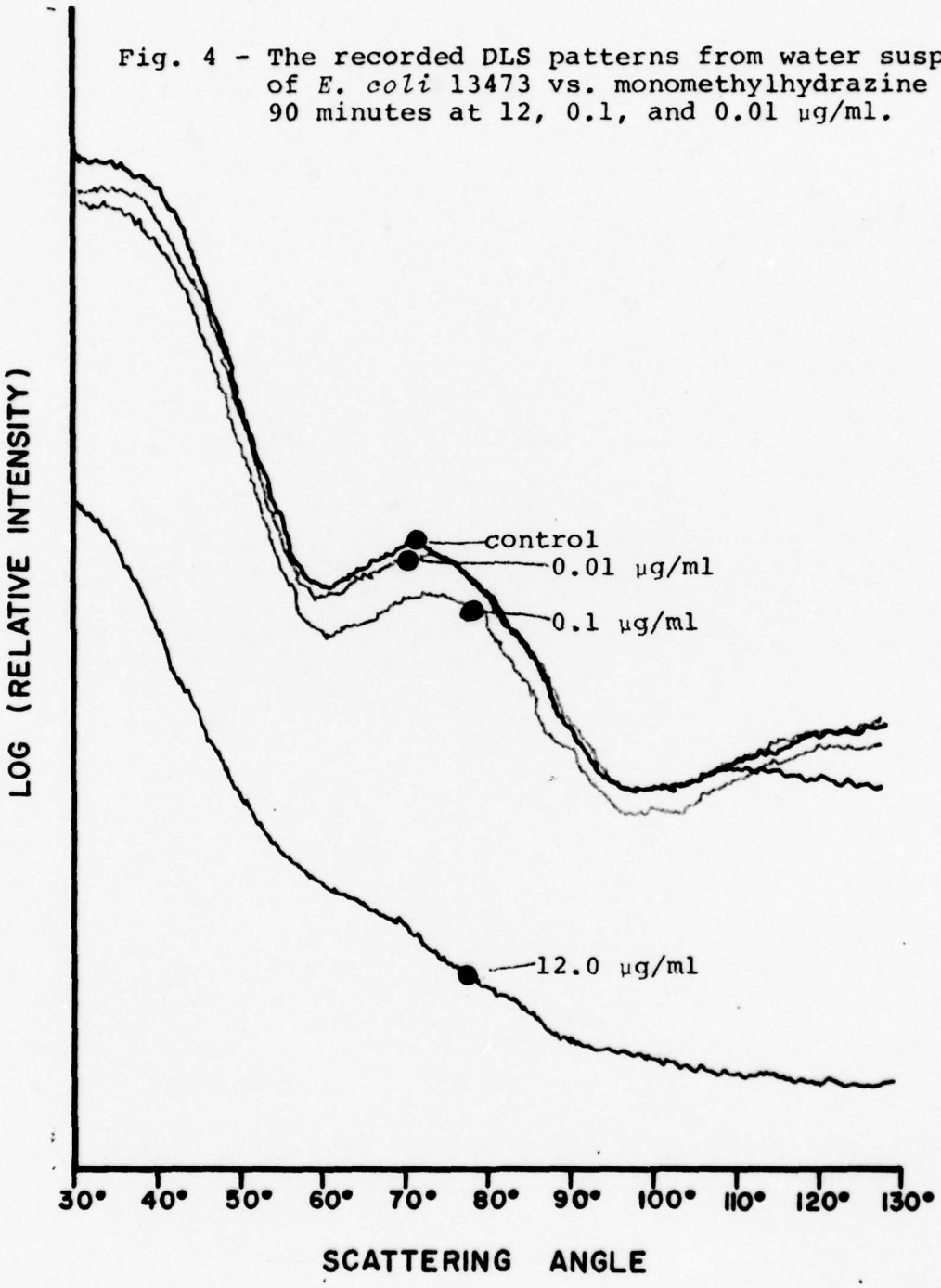


Fig. 4 - The recorded DLS patterns from water suspensions of *E. coli* 13473 vs. monomethylhydrazine after 90 minutes at 12, 0.1, and 0.01 µg/ml.



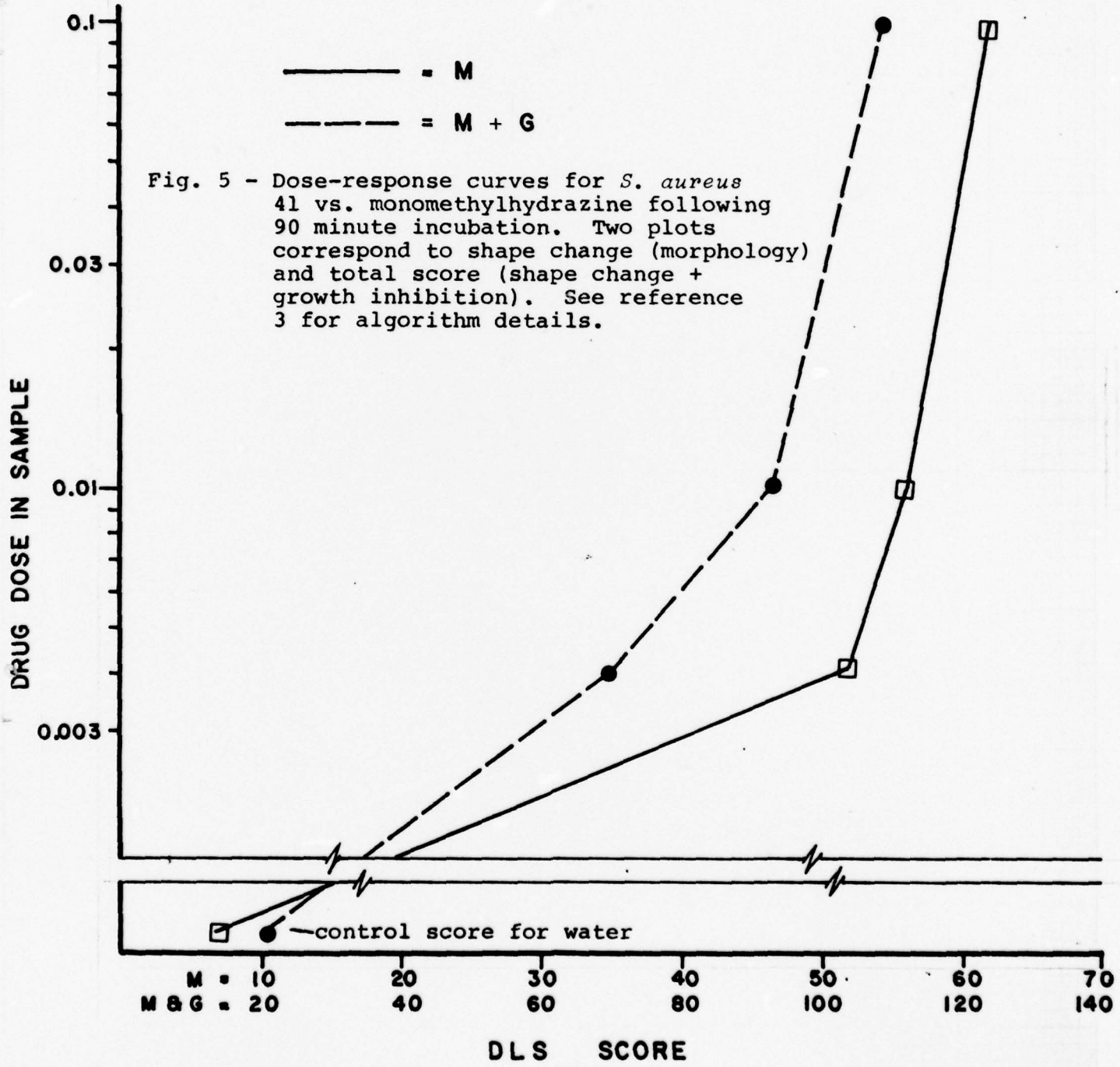


Fig. 5 - Dose-response curves for *S. aureus* 41 vs. monomethylhydrazine following 90 minute incubation. Two plots correspond to shape change (morphology) and total score (shape change + growth inhibition). See reference 3 for algorithm details.

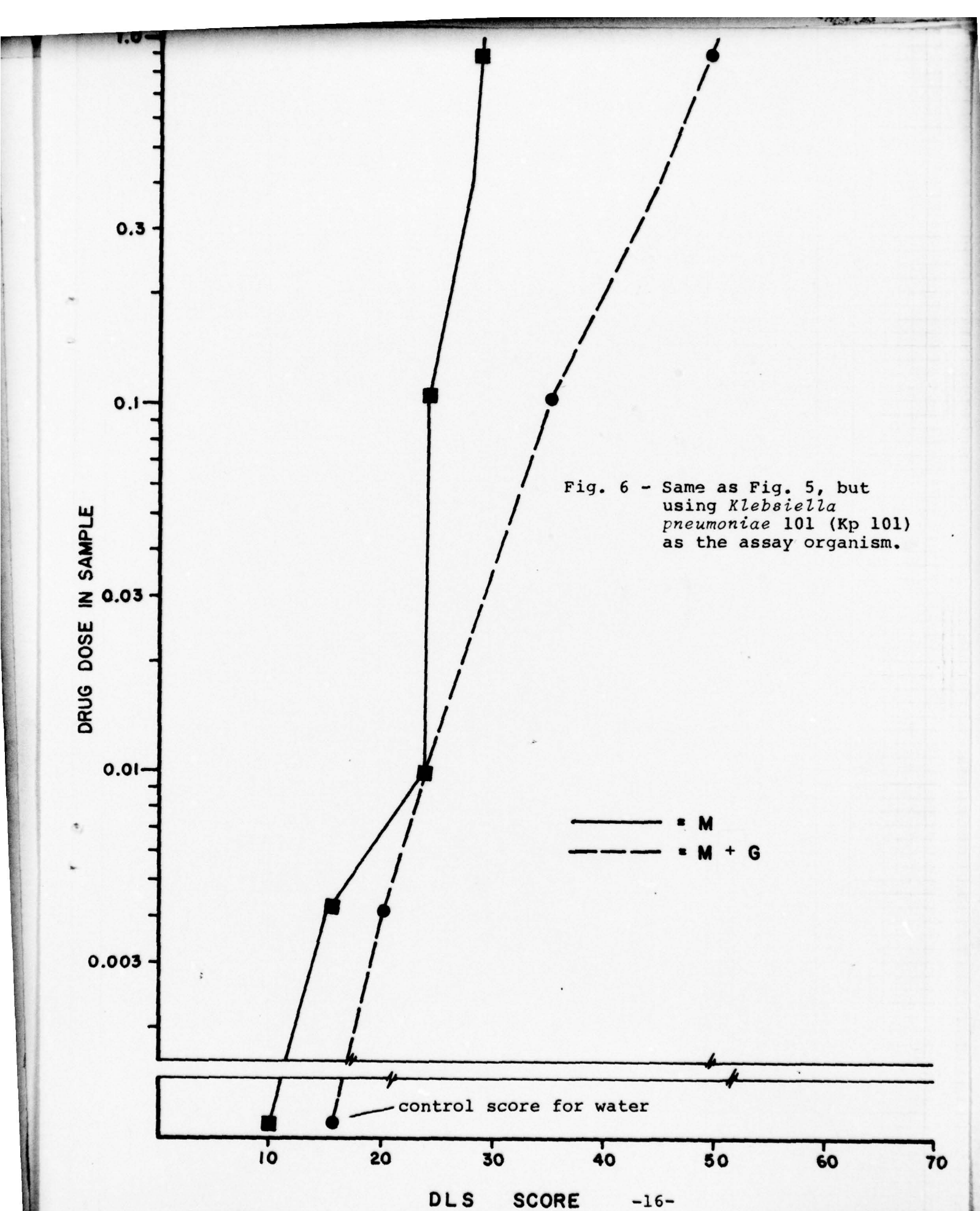


Fig. 6 - Same as Fig. 5, but using *Klebsiella pneumoniae* 101 (Kp 101) as the assay organism.

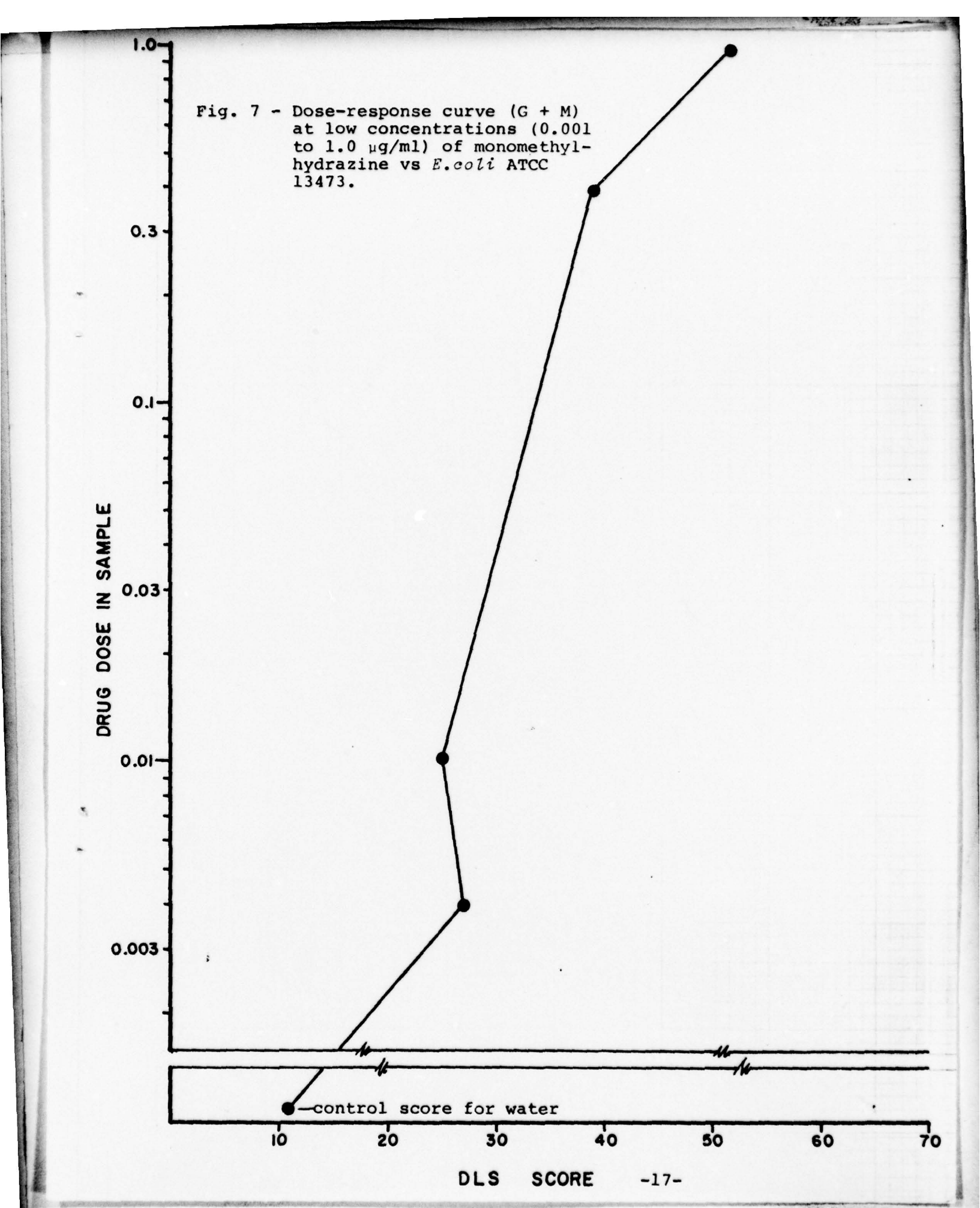
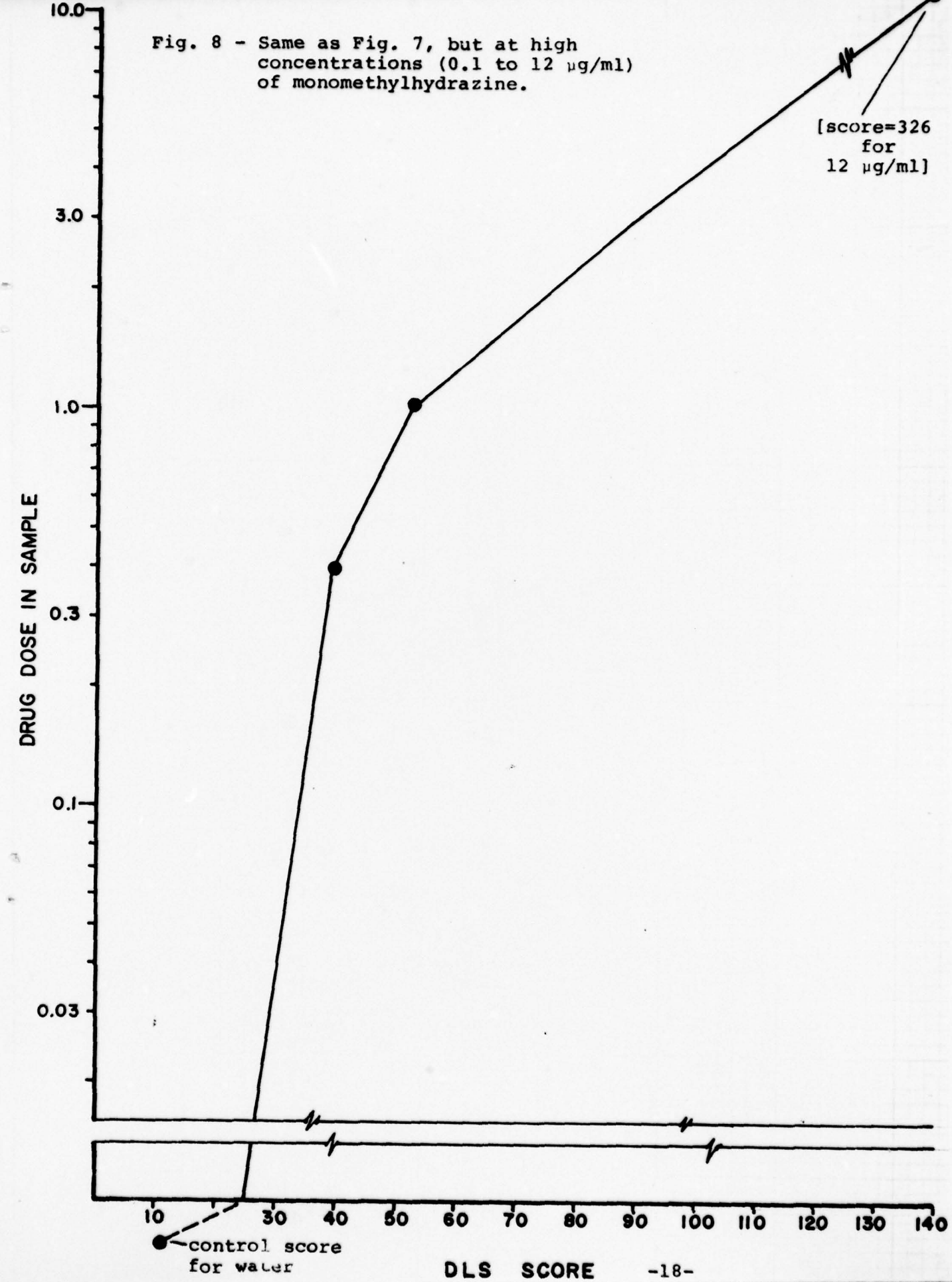


Fig. 7 - Dose-response curve (G + M) at low concentrations (0.001 to 1.0 µg/ml) of monomethylhydrazine vs *E.coli* ATCC 13473.

Fig. 8 - Same as Fig. 7, but at high concentrations (0.1 to 12 µg/ml) of monomethylhydrazine.



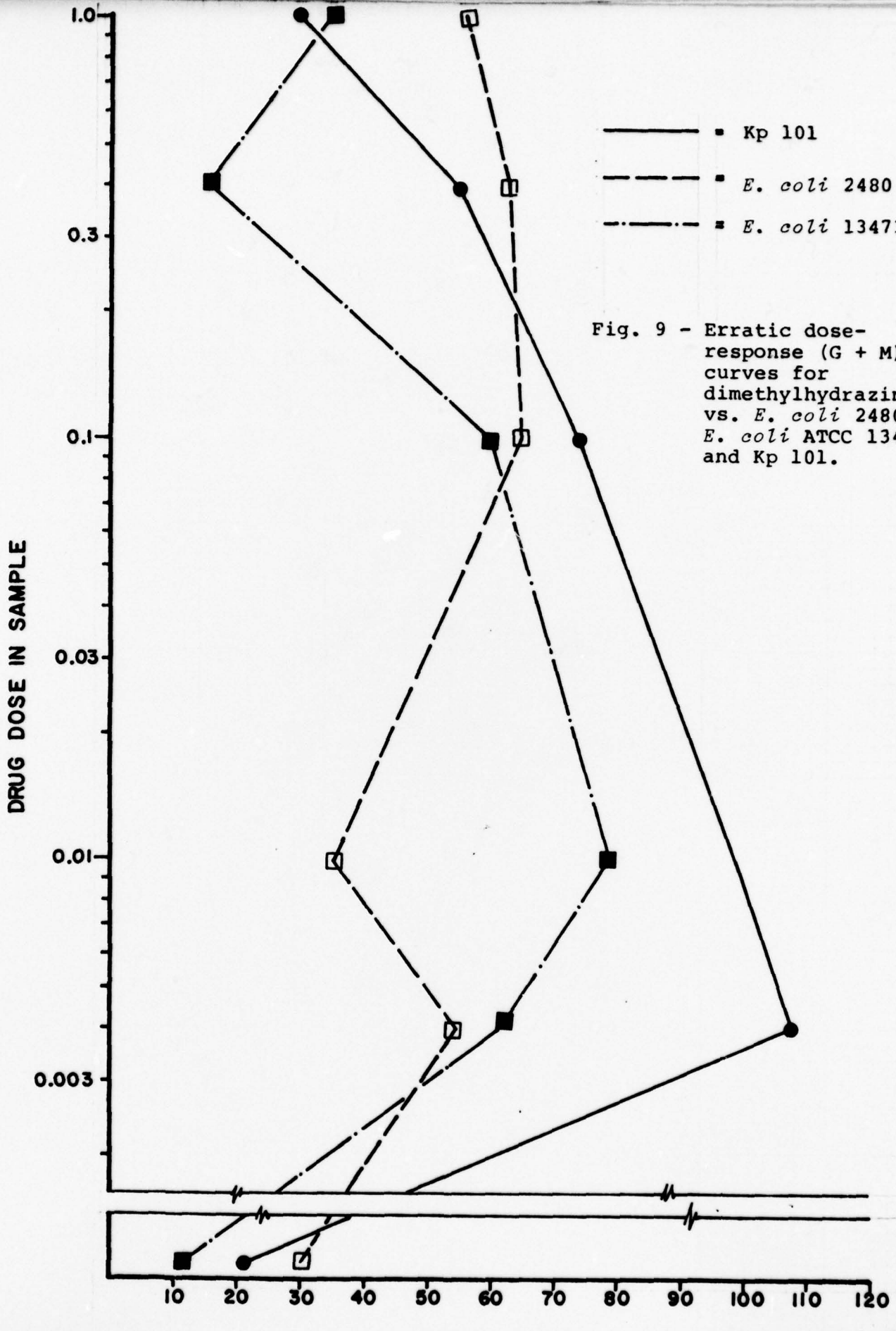
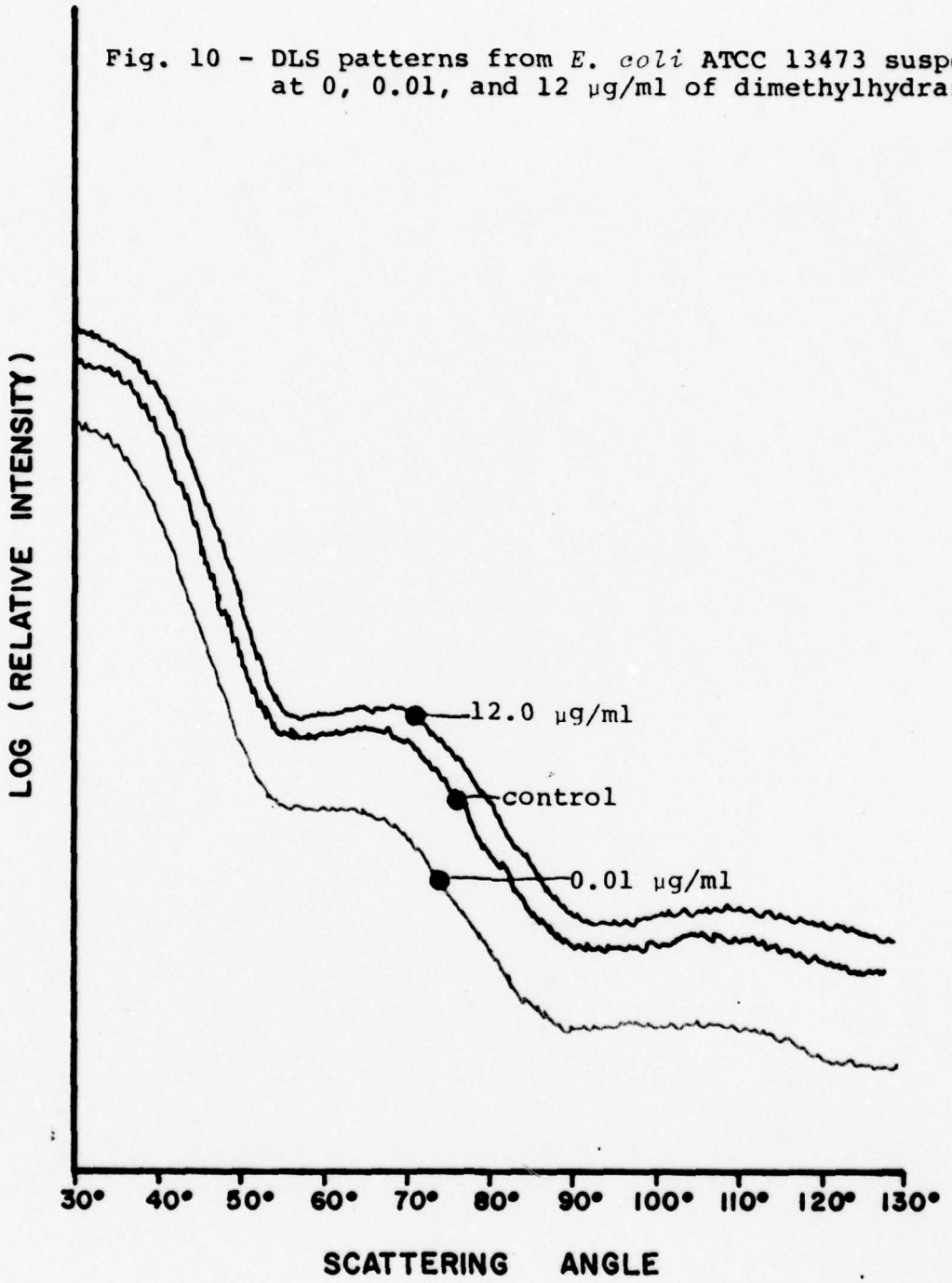


Fig. 9 - Erratic dose-response (G + M) curves for dimethylhydrazine vs. *E. coli* 2480, *E. coli* ATCC 13473, and Kp 101.

Fig. 10 - DLS patterns from *E. coli* ATCC 13473 suspensions at 0, 0.01, and 12 µg/ml of dimethylhydrazine.



responsive. E. coli 3643 exhibited sensitivity to NDMA to 6.0 µg/ml, but the growth curves were not sharp. An attempt to improve the morphology effects on E. coli 3643 over 3 hours using synthetic medium instead of water, or additional brain heart infusion broth (BHI) in the water failed. The best results were obtained in pure water. E. coli B-8 and Kp 886 also yielded poor DLS scattering curves for the bacteria after 3 hours of incubation and the results did not indicate that further modifications of the protocols for these bacteria would be worthwhile. E. coli 2480 without added purines nor amino acids was "responsive" but by stimulation of growth rather than by inhibition to a sensitivity of at least 0.6 µg/ml. Addition of purines and amino acids almost completely reversed this effect. Similarly for Kp 101, the addition of purines and amino acids completely reversed the effect of NDMA. Similar results were achieved with the addition of inositol and choline.

Although E. coli 2480, E. coli 13473 and perhaps S 41 as well as K 886 deserve further effort, with modified protocols vs. NDMA, the best assay organism for this chemical so far is Kp 101 per Fig. 11. Details of culture growth conditions, including (i) volume of fresh broth to volume of overnight culture, (ii) temperature, (iii) time for incubation with chemical, and (iv) pH control, require considerable attention to insure reproducible results on a daily basis. The nature of the problems encountered are shown in Figs. 12 and 13 where "morphology" and "growth" in the presence of chemical are seen to be functions of growth stage of the bacteria and length of incubation before assay.

JP4 and JP8

Figures 14 and 15 show the DLS patterns, produced by the WSF of JP4 and JP8, respectively, at various concentrations following 90 minutes of incubation. Figures 16 and 17 are corresponding data for 120 minutes. These data were generated using a new Differential III system which incorporates a detector array. The extensive on-board computer system permitted a detailed Chebyshev polynomial analysis yield, a direct measure of growth inhibition (stimulation), and a more definitive analysis of morphology differences. Note that the fuel WSFs seem to stimulate growth slightly. In general, the curves show that the size distribution of fuel-affected cells are broadened with increasing concentration. It is estimated that the maximum WSF is about 0.01 µg/ml and the lowest concentration detected was about 0.001 µg/ml. Table 1 presents the dose-response data of the % growth inhibition (I) and shape (morphology, M) change for the two fuels at 90 minutes. Note that the negative growth inhibition values correspond to growth stimulation.

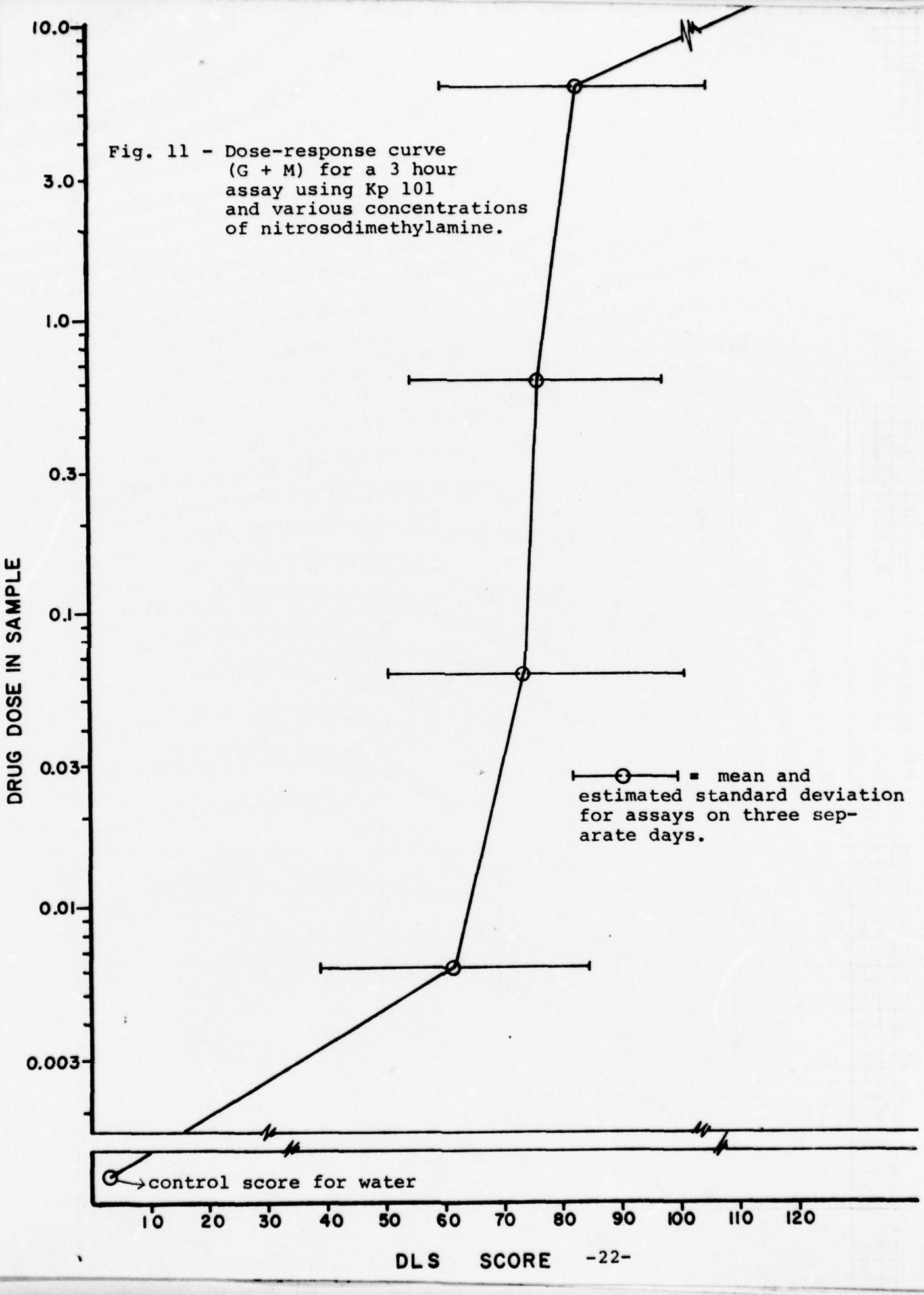


Fig. 11 - Dose-response curve (G + M) for a 3 hour assay using Kp 101 and various concentrations of nitrosodimethylamine.

Fig. 12 - DLS patterns recorded on successive days from water suspensions of Kp 101 vs. nitrosodimethylamine.

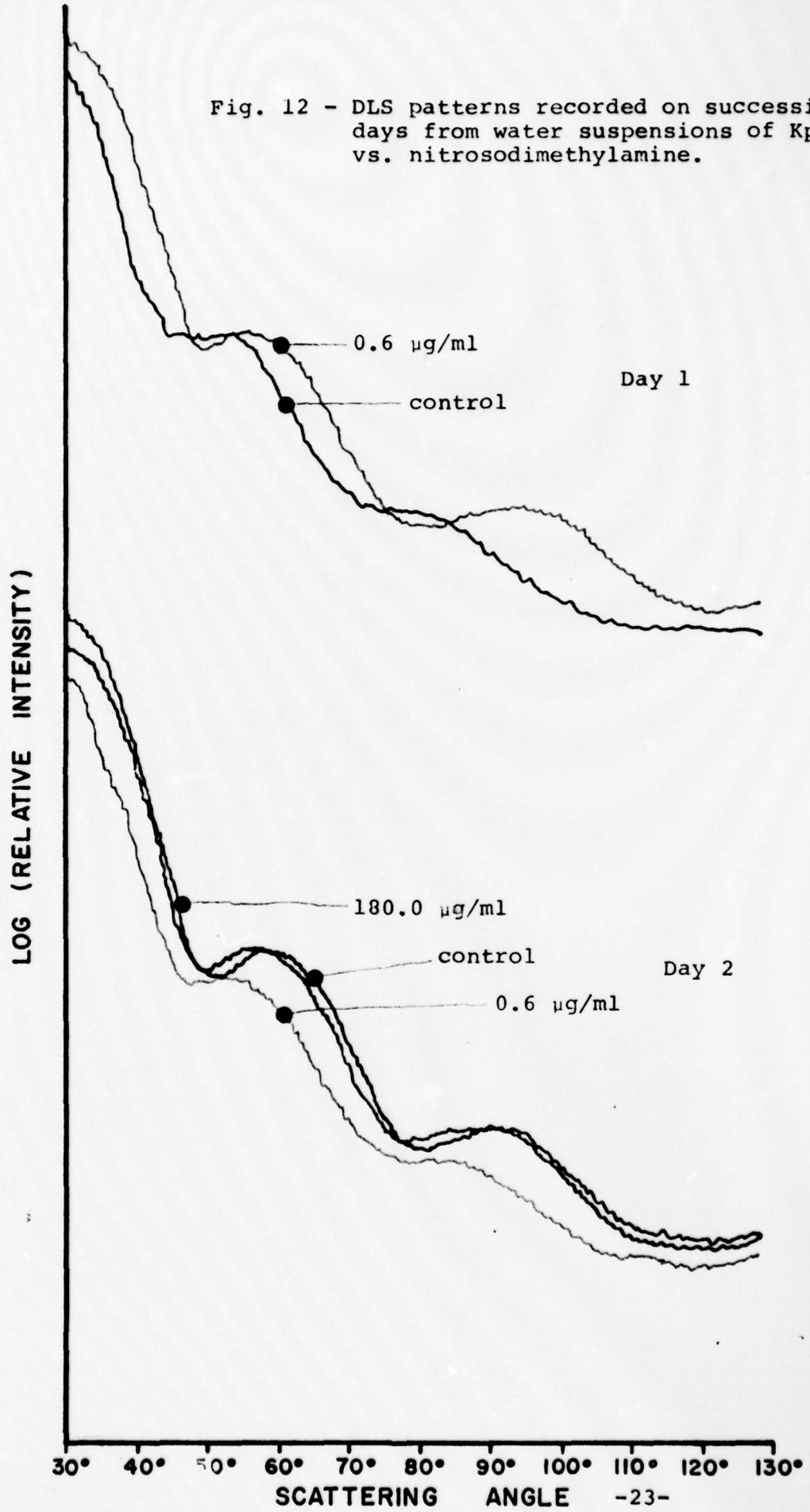
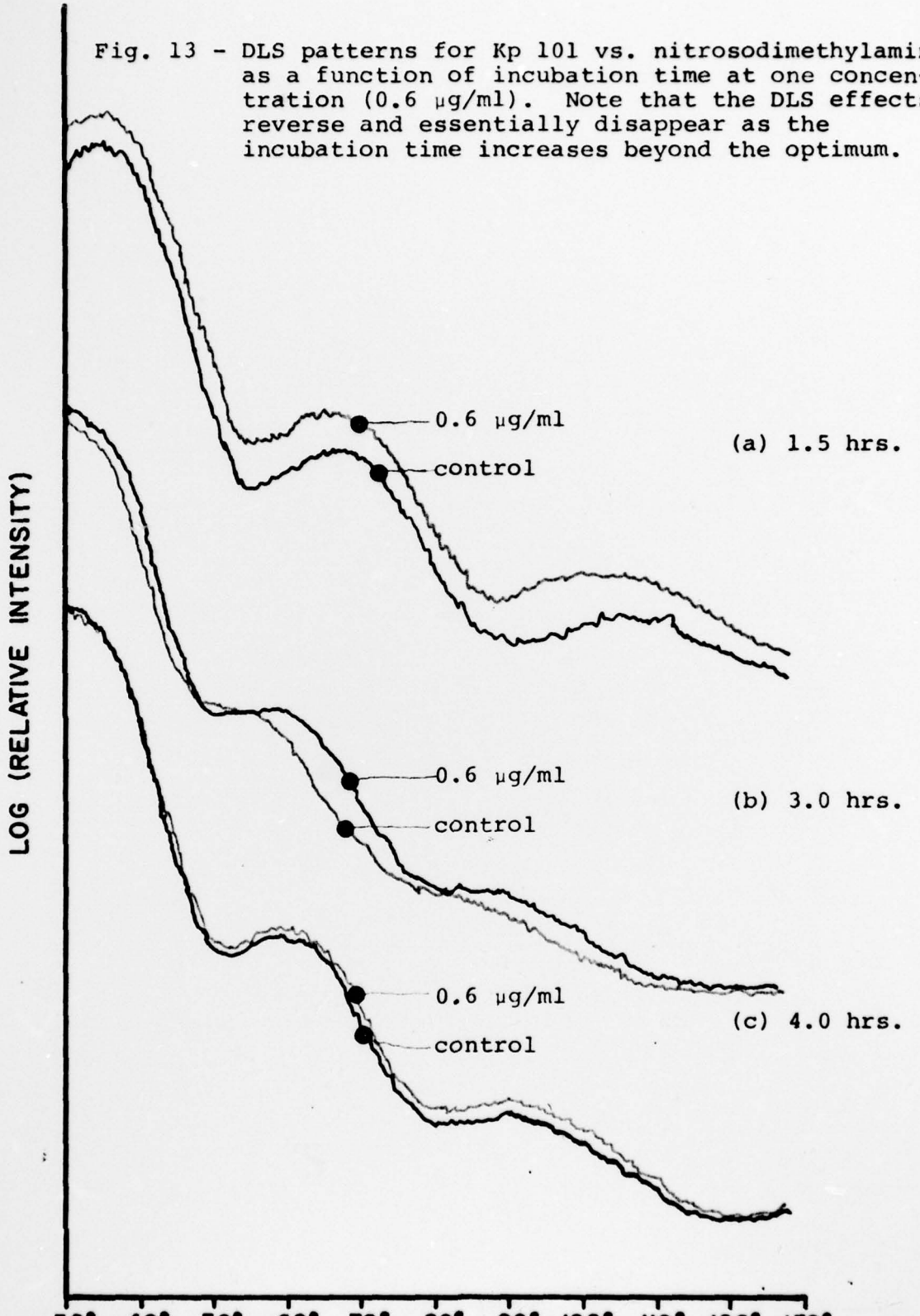


Fig. 13 - DLS patterns for Kp 101 vs. nitrosodimethylamine as a function of incubation time at one concentration (0.6 µg/ml). Note that the DLS effects reverse and essentially disappear as the incubation time increases beyond the optimum.



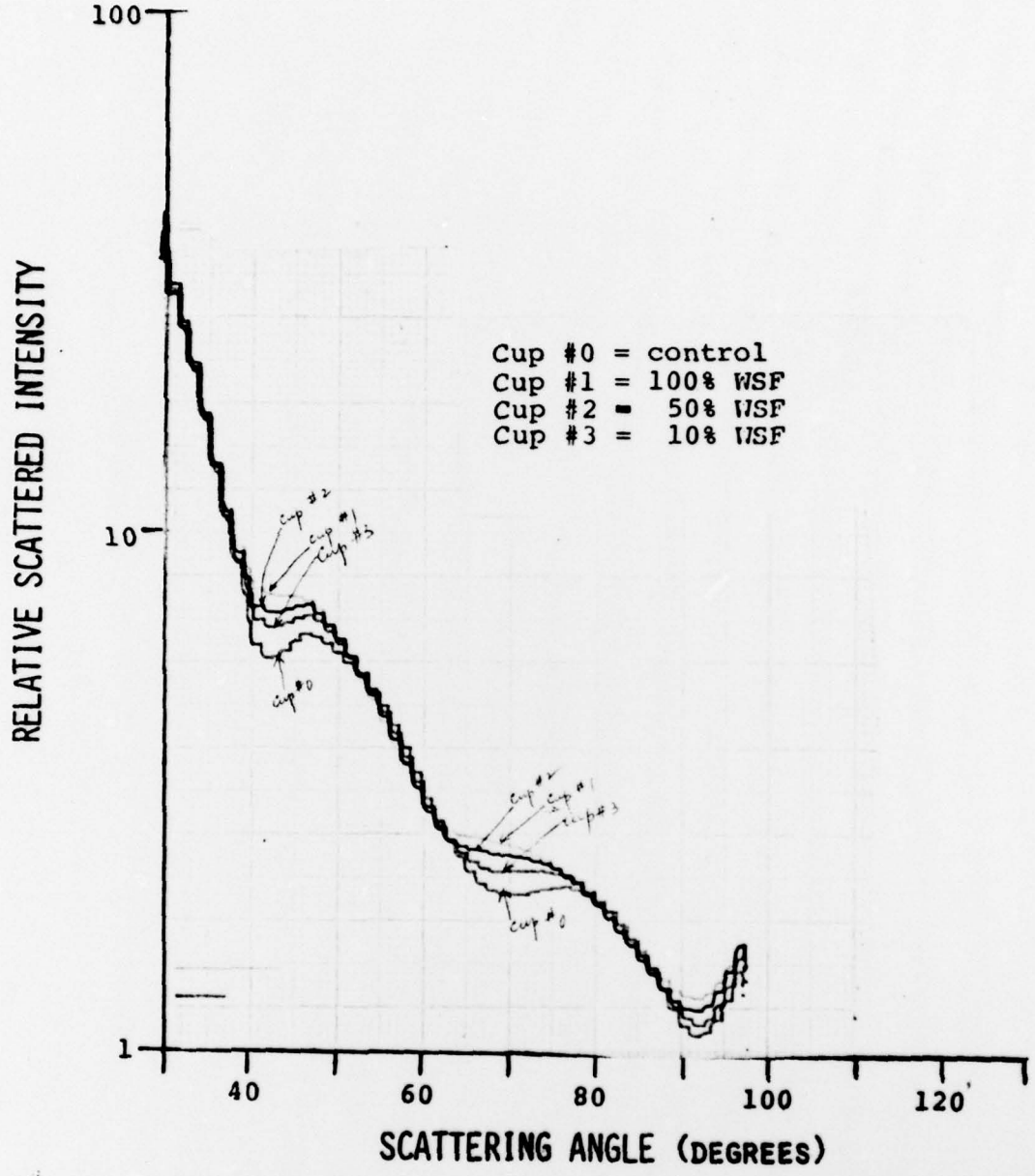


Fig. 14 - DLS patterns recorded at various dilutions (100%, 50%, 10%) of the water soluble fraction (WSF) from JP4 against *S. aureus* 41. The measurements were made using a new *Differential III* system incorporating a 15 element array. The system automatically interpolates and plots the continuous DLS patterns as shown. All incubation times are 90 minutes.

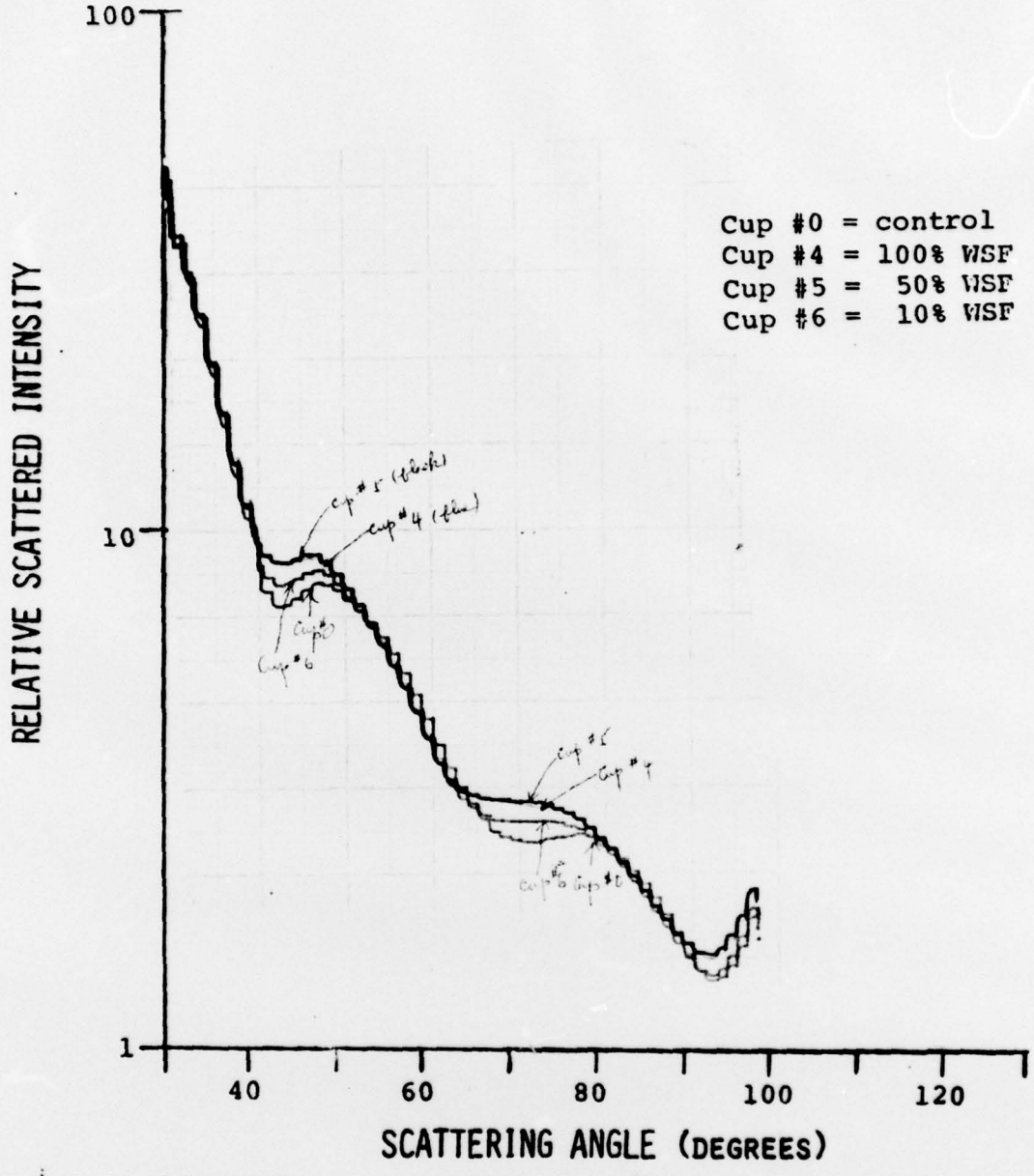


Fig. 15 - Same as Fig. 14, but for JP8.

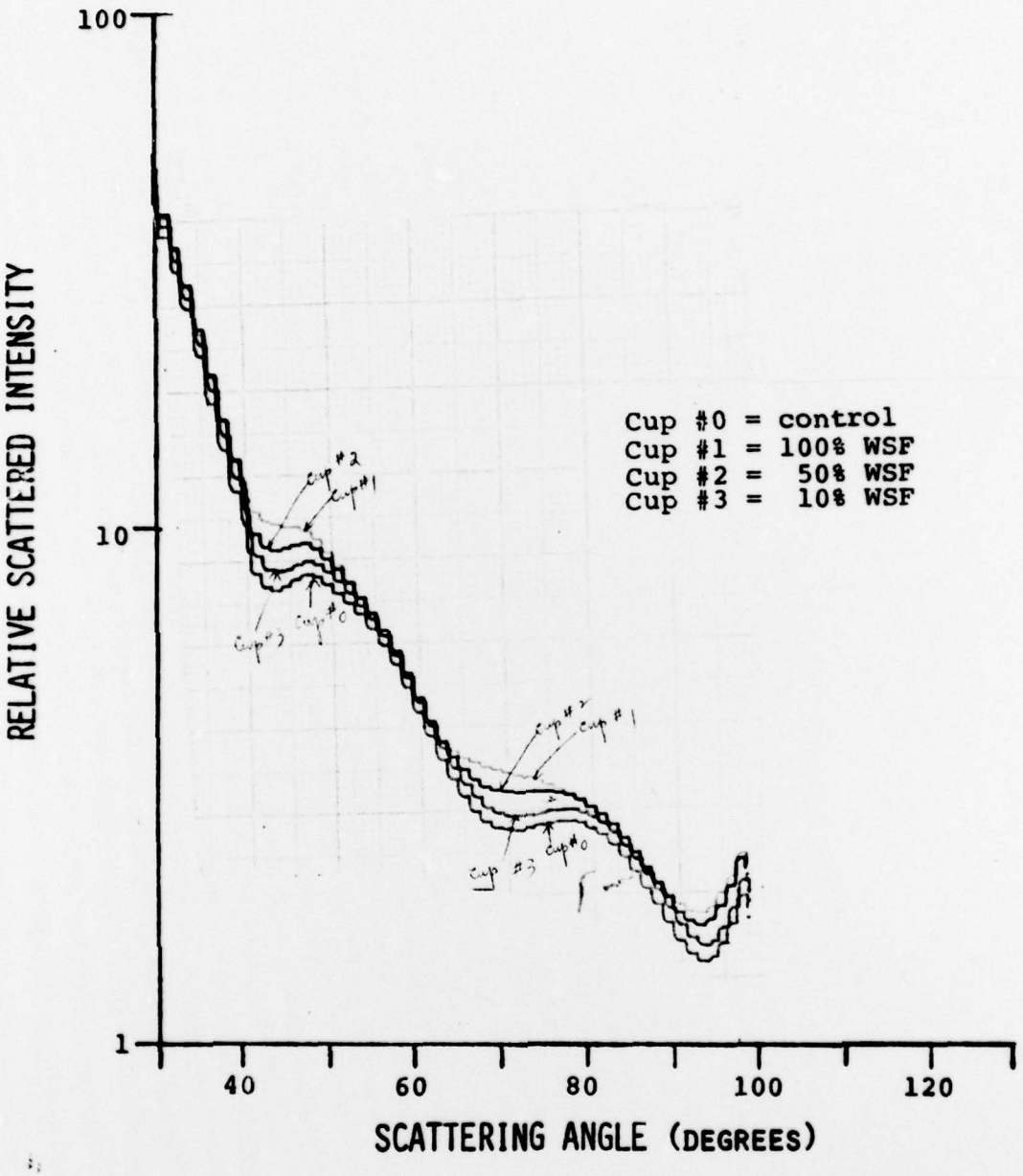


Fig. 16 - Same as Fig. 14, but for 120 minute incubation.

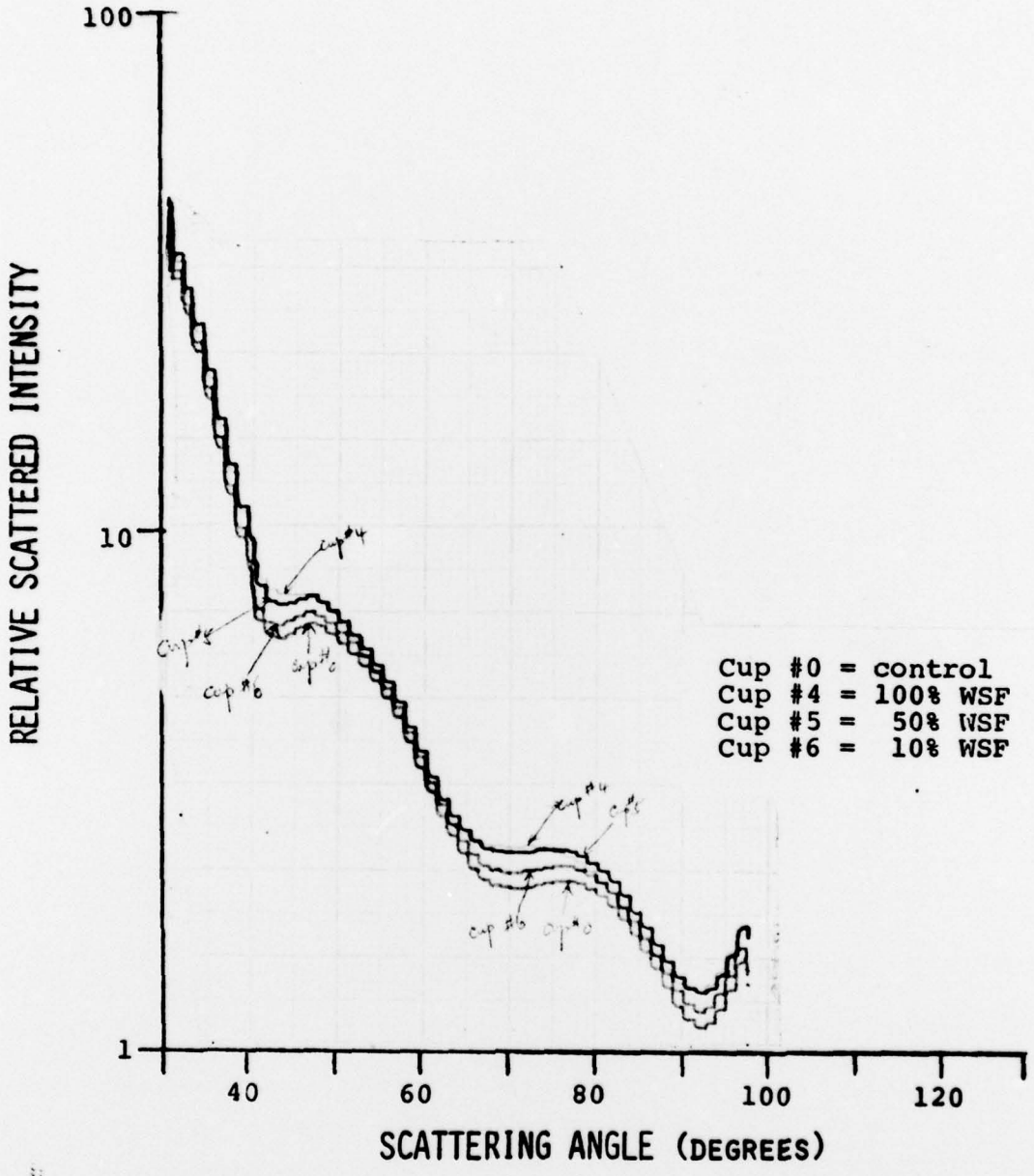


Fig. 17 - Same as Fig. 15, but for 120 minute incubation.

TABLE I
S. Aureus 41

% Inhibition (I) and Shape Change (M) vs. WSF Concentration for JP4 and JP8 at 90 Minutes

% WSF	%I (JP4)	M (JP4)	%I (JP8)	M (JP8)
100	-12	6.6	-13	4.2
50	- 8	3.9	- 9	4.4
10	- 2	.9	.1	1.4

Most of the growth in the predominantly water suspension is endogenous, since the 0.3 ml inoculum in 15 ml corresponds on effective 2% broth solution, but it is interesting to note that after 90 minutes the ratio of bacteria to the number at time zero was about 2.4. At 120 minutes, this value rises to 3.

ABSOLUTE CONCENTRATIONS OF CELLS

We have previously discussed the stability of the Bactisource™ and the fact that we may determine absolute numbers for each strain by reference to a single Petroff-Hauser counting chamber measurement. By making a single plot at the beginning of each day, its comparison with the similar plot of a previous day for which a Petroff-Hauser count has been made, permits a deduction of the absolute cell numbers present. Figure 18 shows the variation of the DLS pattern from successive samples made over a period of several hours from 0.3 ml of Bactisource™ - generated E. coli ATCC25922 cells added to 15 ml distilled water. At t=0 the Petroff-Hauser count was 9×10^7 cells/ml. Thus adding 0.3 ml to 15 ml of distilled water yielded $0.3 \times 9 \times 10^7/15.3 = 1.76 \times 10^6$/ml. At t = 4 hrs, the DLS pattern has shifted downward 0.25 inches on a scale corresponding to one decade per 3 inches displacement. Thus at 4 hours, the number of cells in the Bactisource™ - generated suspension is just $1.76 \times 10^6 \times 10^{.25/3} = 2.13 \times 10^6$/ml. Such graphical analysis is not required in the new Differential III systems since these generate directly the Chebyshev coefficient expansions of the DLS patterns in sin θ/2. Thus

$$\ln [I(\sin\theta/2)] = \sum_{n=0}^{N} a_n T_n [\cos(\sin\theta/2)] \qquad (2)$$

If the coefficients a_n correspond to those generated at the time of a Petroff-Hauser count and b_n correspond to the coefficients generated on some subsequent day, then the ratio of cell densities on the subsequent day to that on the day of the Petroff-Hauser calibration is simply

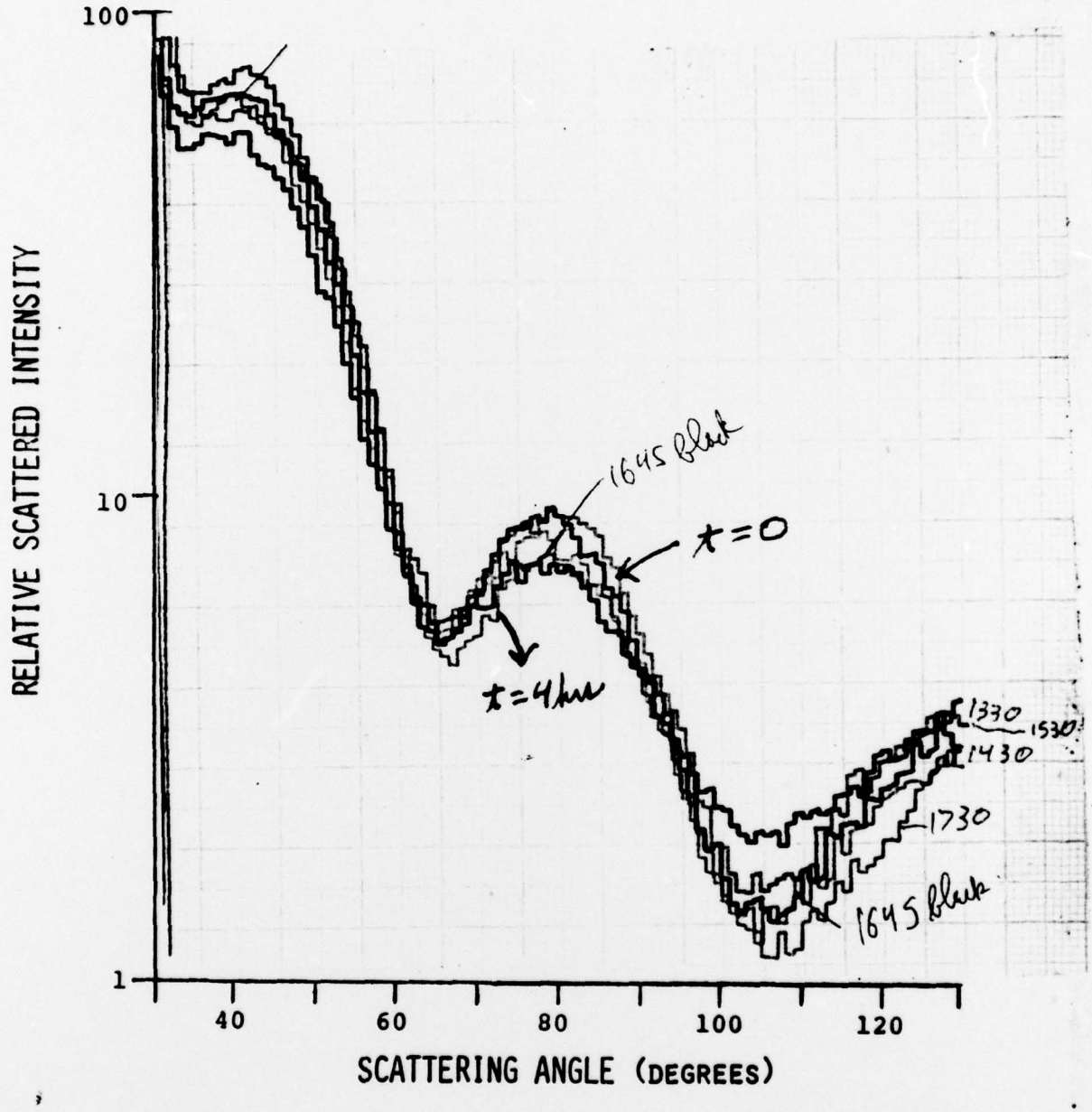


Fig. 18 - Superimposed DLS patterns from *Bactisource(TM)* generated suspensions of *E. coli* ATCC 25922 cells spanning a period of several hours. Variation of cell densities over four hours was less than 20%.

$$R=n/n_o = \exp [1/2(b_o-a_o)], \quad (3)$$

where a_o and b_o are the corresponding zero-order Chebyshev expansion coefficients.

It should be noted that each strain used must have its own Petroff-Hauser count performed at least once, must be generated under constant Bactisource™ conditions prescribed by Eq. (1) with $2F/(\alpha V) < 1$, and must produce a DLS pattern of a shape nearly identical to that corresponding to the Petroff-Hauser counted suspension.

MIXED CULTURES

Figure 19 presents the superimposed DLS patterns from three suspensions; S. aureus 41 (0.15 ml overnight culture in 15 ml DW), E. coli, CDC 2051 (0.30 ml overnight culture in 15 ml DW), and the mixture of the two (0.15 E. coli plus 0.075 ml S. aureus). Figure 20 illustrates the DLS pattern attained for a constant S. aureus inoculum (0.15 ml in 15 ml DW) and various concentrations of E. coli (from 0 to 0.15 ml). The presence of appreciable concentrations of a second bacterial strain is thus seen to degrade the initial DLS pattern and thereby degrade the DLS assay itself. Since endogenous growth will permit, at best, an increase of cell concentration of only a factor of two or three during a typical 90 minute assay, an assay based on changes in DLS patterns of a mixed culture, only one of whose constituents is sensitive to a particular toxicant class, will not be as sensitive as a DLS assay using a pure culture. If the two constituents started at equal concentrations, the unaffected strain would overwhelm the affected strain in the case of toxicant inhibition, while for growth stimulation, both strain contributions would be comparable per Fig. 19.

The most effective means of using several strains simultaneously for purposes of screening several classes of toxicants is to keep them segregated. This procedure is incorporated into the DLS 800 system whereby five different strains may be run in parellel to identify and quantify up to 30 different classes of compounds. Assay strain purity is, therefore, a necessity for the practical implementation of the DLS bioassay technique.

ASSAY RESPONSE TIME

The general procedures explored in the present study for the application of DLS techniques to the detection of toxicants in waste waters require that an aliquot of exponential phase bacteria be added to the waters. The bacteria should be produced by a chemostatic device at an essentially fixed concentration in broth. The final broth/water ratio is of the order of 2%. At this level of nutrient, maximum bacterial concentrations after 24 hours will

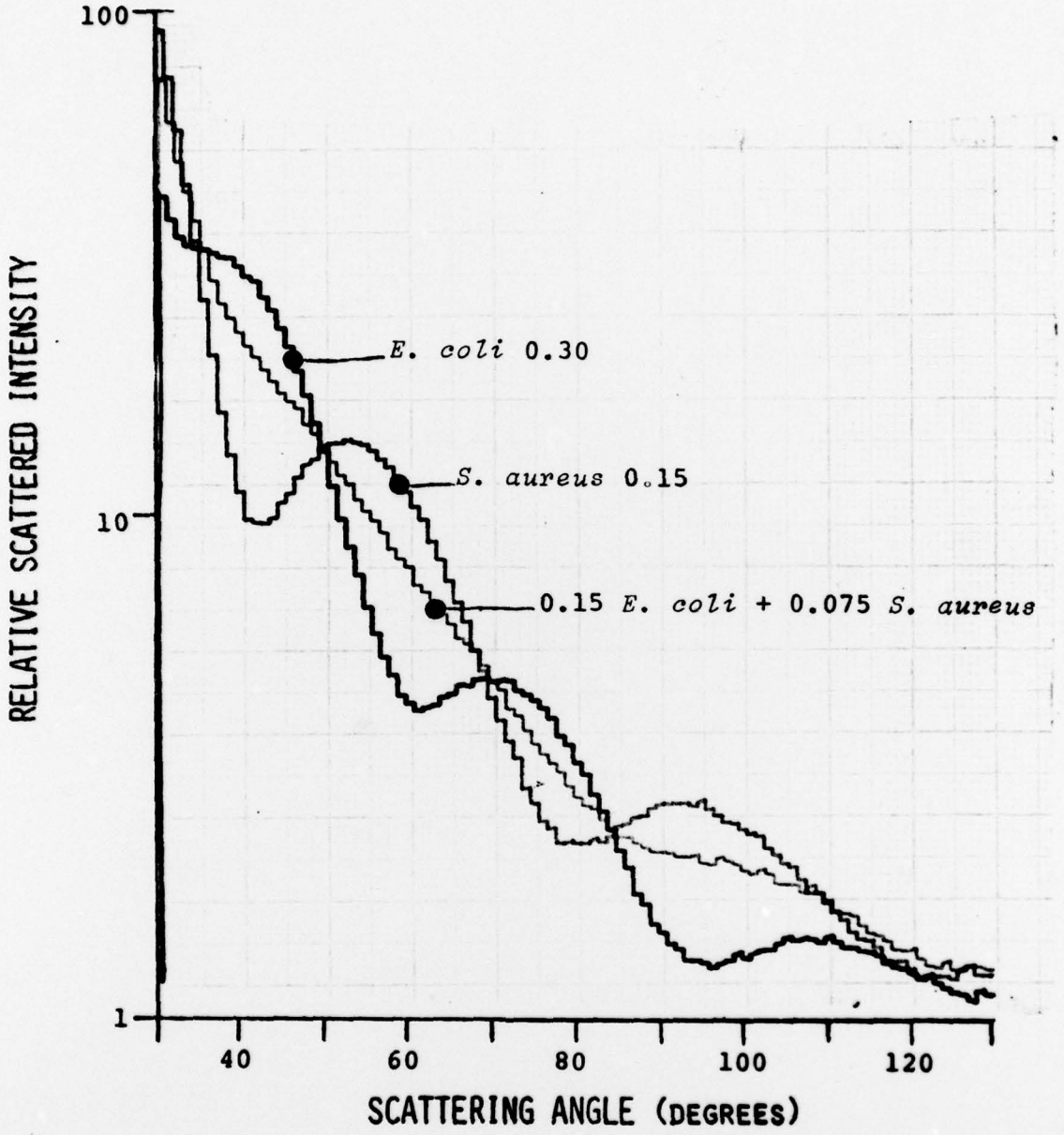


Fig. 19 - DLS patterns from pure cultures of *E. coli* CDC 2051 (0.3 ml in 15 ml DW) and *S. aureus* 41 (0.15 ml in 15 ml DW) together with the DLS pattern of a mixture of the two species (0.15 ml *E. coli* + 0.075 ml *S. aureus*).

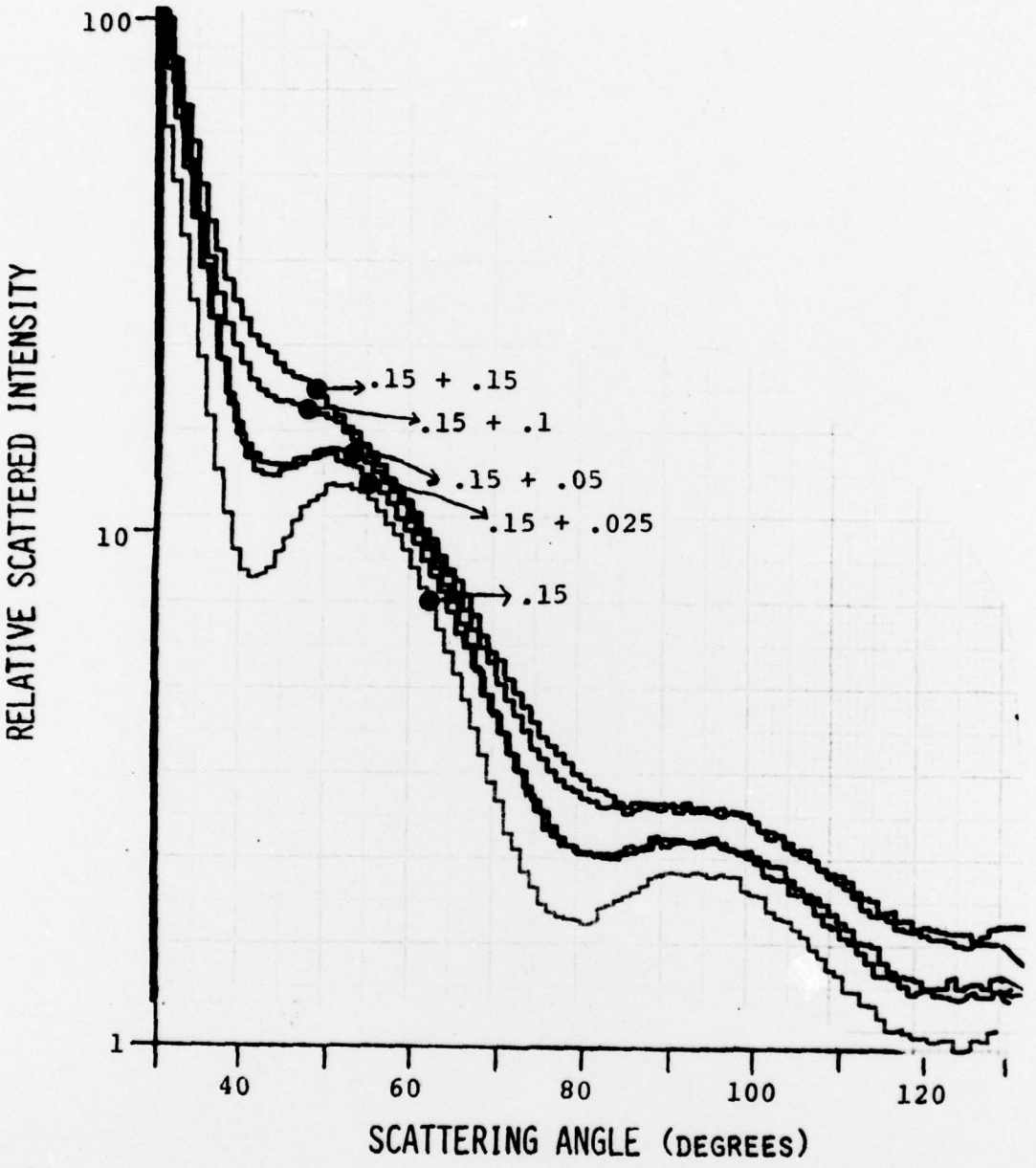


Fig. 20 - DLS patterns from water suspensions of *S. aureus* 41 to which have been added various concentrations of *E. coli* CDC 2051.

probably be less than 5×10^7/ml if the solutions are inoculated at 2×10^6/ml. Indeed, for the most rapidly growing species studied in the current program, by 120 minutes after inoculating the 15 ml water specimens, the bacterial number densities had only increased by about a factor of 3. Within 24 hours, an increase of cell numbers greater than an order of magnitude would not be realistic since, by that time, many cells will have started to lyse in the nutrient depleted medium. Thus an upper limit of about 24 hours might be established for the DLS assay, were it not for the so-called MIC effect.

The minimum inhibitory concentration (MIC) of a particular toxicant is defined as the least concentration inhibiting bacterial growth after 24 hours of exposure. Figure 21 presents a schematic set of idealized dose-response (log C vs. S) curves for a particular assay strain challenged at different toxicant concentrations. As the interaction time increases, the dose-response curve becomes sharper and eventually breaks at the MIC concentration, C_o. Above C_o, the growth has saturated, while there is effectively no response (or growth inhibition) below C_o. Note that the broadest dynamic range of concentrations will be detectable within the early times of the assay. At subsequent times, the lower levels of toxicants have no effect relative to the control. These effects are vividly illustrated by the experimental data of Fig. 13. For toxicants producing growth stimulation, such action is again time limited because of the finite nutrient supply available; the controls will eventually catch up to the stimulated cells. At very short times, of course, low toxicant levels will not be able to produce measurable effects. It remains, therefore, to define an optimal assay time.

The contract objective for confirmation of a rapid bioassay precludes any periods greater than a few hours. Since the maximum DLS changes occur during periods of rapid bacterial growth and since, furthermore, the simplest protocols require no preparations or modifications of the waste waters other than filtering, the optimal assay time has been established at 90 minutes. For some classes of toxicants such as the nitrosoureas an additional 90 minute period may be required to achieve reasonable sensitivity and linearity. If toxicants are present at very high levels, then early reporting of their presence is possible in considerably shorter times. For the strains examined during the present study, the minimal assay time for the detection of high toxicant loads is 20 minutes, or the equivalent of about a single bacterial generation time.

RECOMMENDATIONS FOR FUTURE WORK

The DLS bioassay for the detection of toxicants in waste waters has been successfully demonstrated. The sensitivity of the method (SOM) must now be compared and correlated with the more

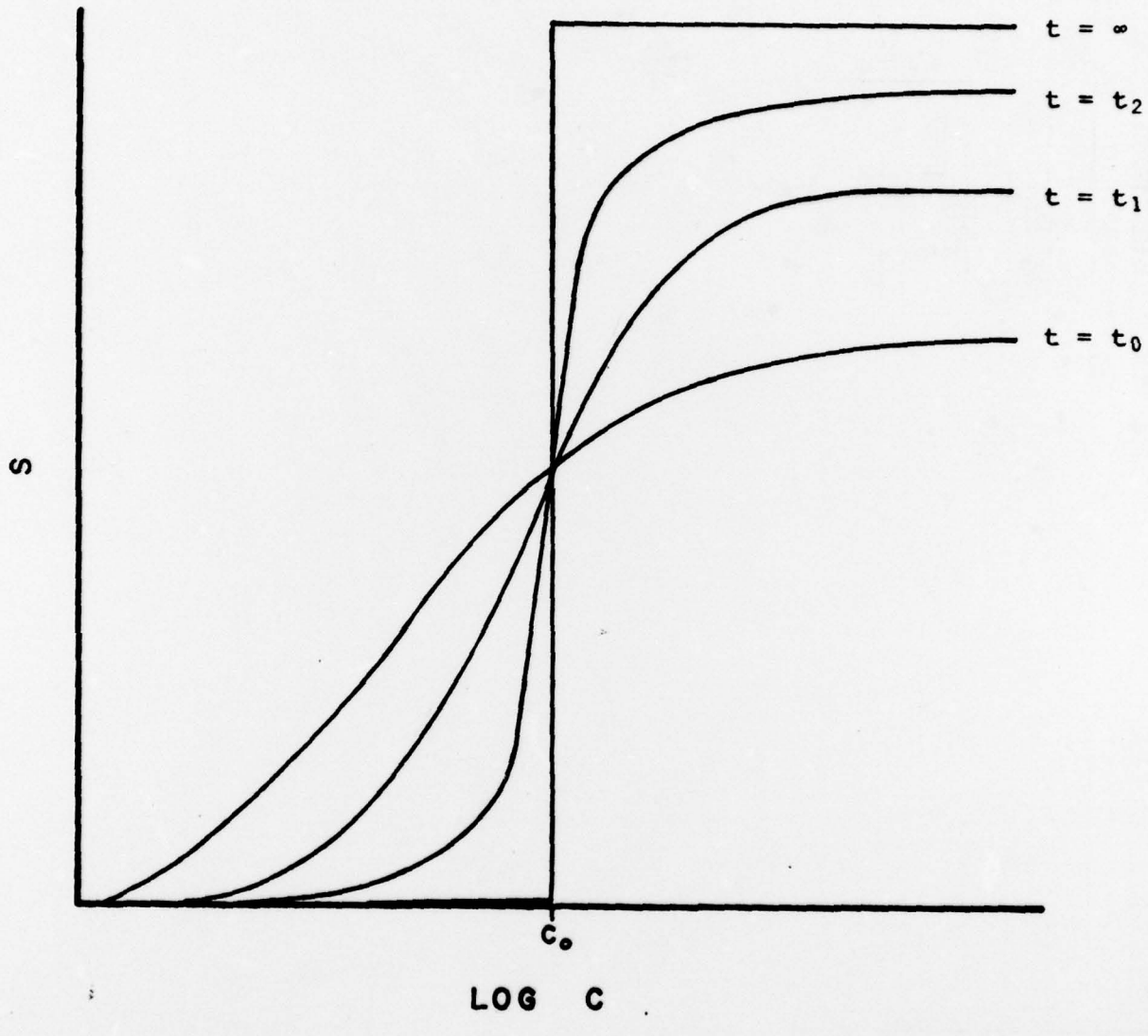


Fig. 21 - Theoretical variations of dose-response curves [log C vs. 5] with time.

conventional fish bioassay. Since the DLS bioassay is expected to be a far more sensitive, rapid, and economic tool than the fish bioassay, work on the latter technique should be phased out nationally as the correlation data becomes overwhelmingly convincing. A scientifically thorough correlation program could be completed within a year using a fully automated DLS 800 system. Such a definitive program should cost about $250,000 in 1979 dollars and would result in substantial savings within a few months of its successful completion by permitting the immediate termination of the wasteful research programs concerning fish and other high life forms.

Continued research funding of the fish bioassay technique in view of the results of the present mini-study may be both wasteful and impractical. There are enormous political pressures within EPA and the Air Force, however, to retain the status quo and continue emphasis on bioassay techniques using higher life forms such as fish. Most scientists would agree that the practical implementation of a fish bioassay for the routine screening of waste waters will not be possible. Nevertheless, an important question remains, viz. are there any important toxicants whose presence can only be detected at meaningful levels by higher life forms such as fish. Plans to expand fish bioassays are currently proceeding and many new industries based on the care and maintenance of such fish are being created, irrespective of the lack of data confirming the unique detection properties of fish. EPA has done little to encourage new approaches and it appears now to rest firmly with the Air Force to try to break this endless chain. Expansion and the practical implementation of the DLS bioassay should become a high priority for Air Force's Environmental Protection research and development programs.

APPENDIX: SUPPLEMENTARY INFORMATION

A. Publications

No papers were submitted for publication during the present contract year. A shortened version of this report will probably be submitted to an environmental journal within the next few months and reprints sent to the distribution list.

B. Professional Personnel

Most of the work on this program was performed by Dr. Israel N. Rabinowitz and Dr. Philip J. Wyatt. They were assisted by Margaret Nesbitt, M.S. and Fern Fisher, B.S.

C. Interactions

A paper, A rapid bioassay screen for water-borne toxicants using differential light scattering, was presented by Drs. Wyatt and Rabinowitz at the January 15-17, 1979 Review of Air Force Sponsored Basic Research in Environmental Protection and Toxic Hazards, held in Los Angeles.

D. New Discoveries, Inventions, and Patent Disclosures

There were no patentable discoveries or inventions made during the contract period.

E. References (from text)

1. P.J. Wyatt, "Differential light scattering techniques for microbiology," in Methods in Microbiology, J. R. Norris and D. W. Ribbons, eds. (Academic Press, New York, 1973).

2. P.J. Wyatt, R.F. Pittillo, L. M. Rice, C. Woolley, and L. B. Mellett, "Laser Differential Light Scattering Bioassay For Methotrexate (NSC-740)," Cancer Treatment Reports 60, 225-233 (1976).

3. P. J. Wyatt and D. T. Phillips, "Laser Light Scattering Bioassay For Veterinary Drug Residues In Food Producing Animals: 1. Dose response results for milk, serum, urine, and bile," J. of Agricultural and Food Chem. 24, 984 (1976); Ibid with M. G. Scher, M. R. Kahn and E. H. Allen, "2. Preparation and procedures and dose-response studies of drugs in bovine tissues," J. Agri. & Food Chem. 25, 1080 (1977).

4. Differential Light Scattering, Rapid Quantitative Antimicrobial Testing, and the new Programmable Differential III, (Science Spectrum, Inc. Santa Barbara, 1978).

5. V. R. Stull, "Clinical laboratory use of differential light scattering: I. Antibiotic susceptibility testing," Clin. Chem 19, 833 (1973).

6. D. T. Phillips and P. J. Wyatt, "Evaluation of laser bioassay techniques for adriamycin, BCNU, and l-phenylalanine mustard," Arthur D. Little, Inc. NCI subcontract A10423-N01-CM-63823.

7. Application Of Differential Light Scattering Assay To Rapid Screening For Veterinary Drug Residues, FDA Final Report, HEW/FDA Contract 223-77-7028, Oct. 1978 (Science Spectrum, Inc.)